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THE UNIVERSITY OF ALBERTA

METABOLITES OF THE SCLERODERRIS CANKER FUNGUS

by

MARIA DA SOLEDADE CORREIA PEDRAS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

EDMONTON, ALBERTA

FALL 1986

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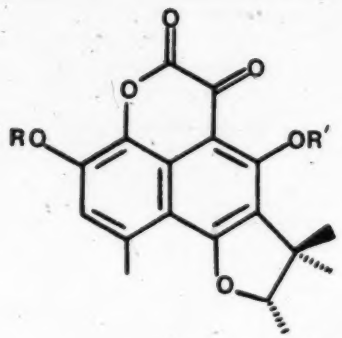
submitted by MARIA DA SOLEDADE CORREIA PEDRAS
in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY

W.F. Hyer
Supervisor

DATE June 6, 1986

ABSTRACT

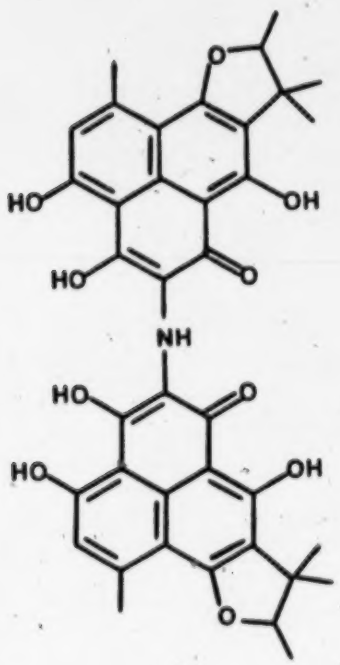
Two new metabolites produced in liquid culture by the fungus Gremmeniella abietina (Lagerb.) Morelet, the causative agent of Scleroderris canker, a serious disease of pines, were isolated and their structures determined. One of these metabolites, a bright yellow compound named scleroderolide, has structure 1 as determined by X-ray crystallographic analysis of its monoacetate 1a. The



1 R=R'=H
1a R=Ac R'=H
1b R=R'=Ac

crystal used for the X-ray analysis proved to be racemic, although scleroderolide (1) showed optical activity. The origin of this discrepancy was investigated. The diacetyl derivative (1b) of scleroderolide showed interesting temperature dependent nmr features.

The structure of the other metabolite (**2**), which we have named Scleroderris green, was determined by analysis of the spectroscopic data of three different pentamethyl derivatives.



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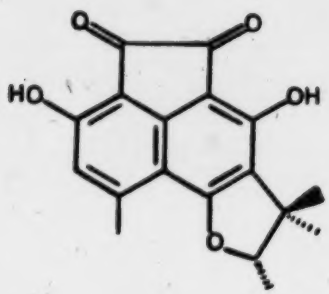
Most strains of the fungus investigated produce phenalenone metabolites. One strain (C656) examined, however, showed interesting differences. The major metabolites produced by this strain are fatty acids and triglycerides.

Biosynthetic studies have confirmed the polyketide origin of the phenalenone metabolites of G. abietina and indicated that sclerodione (3) may be a biogenetic precursor of sclerodin (4), but not of scleroderolide (1).



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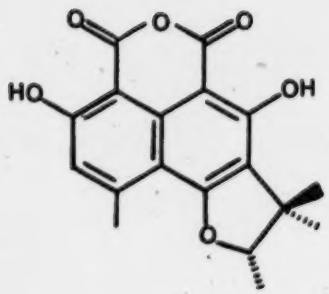

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I. INTRODUCTION

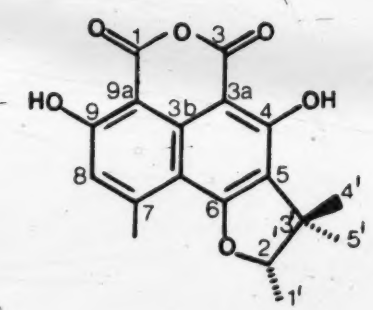
The fungus Gremmeniella abietina (Lagerb.) Morelet (= Scleroderris lagerbergii Gremmen) is a virulent pathogen of pine trees in many parts of the world. In North America the disease caused by this fungus, commonly known as Scleroderris canker, is regarded as one of the most serious tree nursery and reforestation diseases (1). The disease is also prevalent in Europe, where it is known as Brunchorstia dieback (2), and in Japan (3).

Primary infection of the tree is by small, wind-disseminated spores, which are released from fruiting bodies during moist periods. The peak periods of spore dispersal are reached in May through July under North American conditions (4). Usually symptoms develop in the spring of the year following infection, shortly before shoot elongation begins. As shoots on adjacent branches begin to grow, the foliage on infected branches turns grayish green and a yellow-orange discoloration advances from the base towards the tip of the needles. As summer progresses, needles turn yellowish brown and usually drop by autumn. A few months after the branch dies, another fruiting stage of the fungus develops, which then releases spores capable of causing new infections (5). The fungus grows into the main stem of the tree causing a yellowish green discoloration in the inner bark and wood (5). On older trees cankers form which may eventually kill the

tree, whereas young trees often die within a few months. The mechanism by which the fungus kills the tree is not yet known.

The metabolites produced by the fungus Gremmeniella abietina are being investigated (6) in an effort to determine what compounds are responsible for symptom expression and to determine whether there is a correlation between metabolites produced and the pathogenicity of particular strains.

The major metabolite produced by G. abietina, sclerodin (**1**), which crystallizes as pale yellow needles, is the enantiomer of the so-called naphthalic anhydride from atrovenetin (**2**). The enantiomer of sclerodin (**1**),



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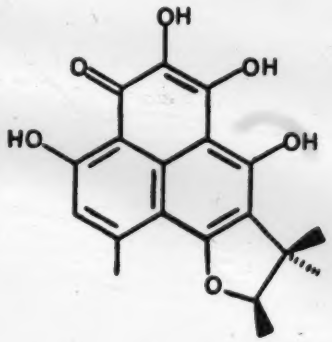
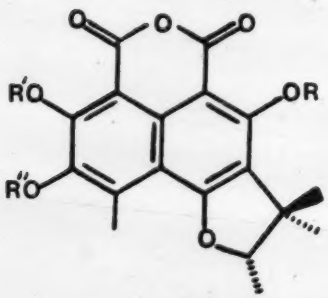


2

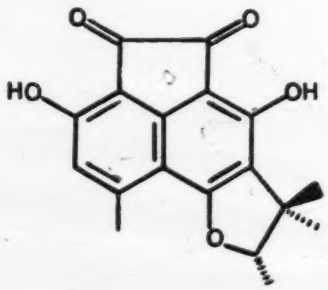
was first obtained by oxidation of atrovenetin (7) and later was isolated from cultures of Penicillium herquei

(8) and subsequently from other fungi (9-11). Sclerodin from G. abietina is levorotatory and has the S-configuration at C-2'.

The mycelium mats of G. abietina are strongly colored: dark green pigmentation with some bright yellow spots. These mycelium mats yield a very dark green extract from which several highly colored metabolites have been isolated (6): 8-hydroxysclerodin (**3**), sclerodione (**4**), Scleroderris blue (**5**), the triketone 6, and a bright yellow compound which is described in the next chapter of this thesis.



3 R = R' = R'' = H
3a R = Me R' = R'' = H
3b R = R' = R'' = Me



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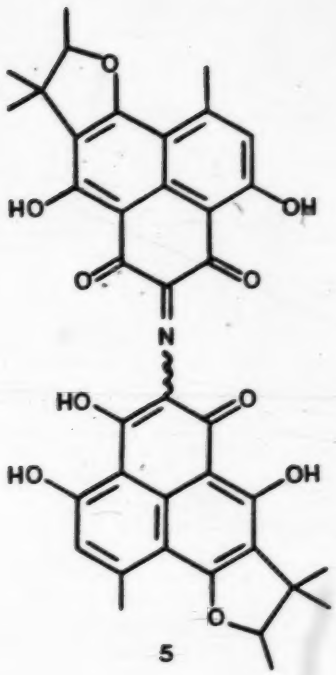
The enantiomer of **6**, atrovenetinone, had previously been prepared either by oxidation of atrovenetin (**2**) with benzoquinone (18), or by photooxidation (12). Ent-atrovenetinone (**6**) is a dark burgundy compound causing a



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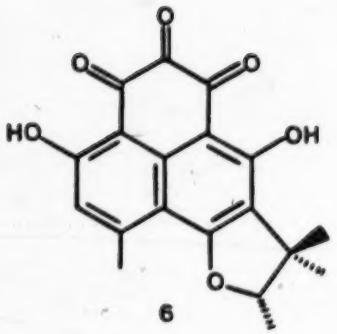

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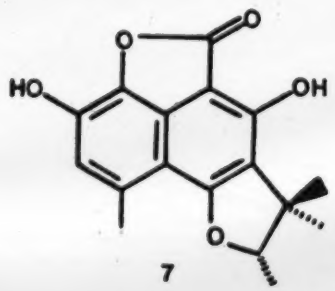
green discoloration on contact with the skin and a blue-green discoloration of paper. It was found (6) that when a solution of 6 is painted on freshly peeled pine the characteristic blue-green color of Scleroderris infected wood developed.

Scleroderris blue (5), the least polar of these metabolites, was rather unstable and difficult to purify. When a solution of this blue compound was allowed to stand in the presence of air, the colorless anhydride 1 was obtained. Analysis of the spectroscopic data of Scleroderris blue suggested the structure 5. Narasimhachari and Vining (8) had previously postulated

that the characteristic green pigment of Penicillium herquei might be formed by a ninhydrin-like reaction between atrovenetinone and amino acids. Structure 5 was confirmed by reaction of ent-atrovenetinone (6) with glycine, which gave the blue pigment in good yield (6).

Scleroderris blue (5) is believed to be responsible, at least in part, for the bluish-green coloration of the wood of Scleroderris infected pine. A compound closely related to Scleroderris blue is also produced by G. abietina. Its isolation and structure determination are described in Chapter III of this thesis.

The structure of sclerodione (4), a bright red compound, was deduced from spectroscopic data and confirmed by alkaline peroxide oxidation. Treatment of 4 with hydrogen peroxide provided the anhydride 1 and the lactone 7. Other oxidation reactions of this diketone are described in Chapter II of this thesis.



7

The more polar component of the crude extract of G. abietina was isolated after treatment of the crude material with diazomethane. Compounds **3a** and **3b** were isolated, and thus it was presumed (6) that compound **3** is produced by the fungus.

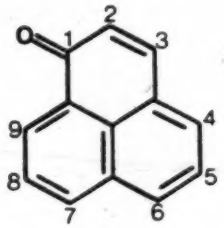
Compounds **1**, **4**, **5**, and **6** have been isolated from two different strains of G. abietina: C699 ("European strain", from New Brunswick) and C708 ("New York strain", from Vermont). Compound **3** was isolated from strain C708.

The taxonomy of G. abietina is not clearly defined. Earlier (13) immunogenic comparisons revealed separate North American, European, and Asian physiologic races. The North American race attacks only young trees. The more virulent European race attacks and kills trees of all ages (5,14). Recently (2,15-18) evidence for further subdivision of G. abietina isolates has been presented.

We have compared (18), using high-pressure liquid chromatography (hplc), the metabolites of four different strains. Interestingly, the metabolites of strain C659 ("North American strain", from Ontario) were very similar to those of C699 ("European strain", from New Brunswick). However the metabolites of strains C706 ("European strain", from Sweden) and C707 ("New York strain". from New York) were quite different from those of strain C699. We have also examined the metabolites of a

new strain, isolated in Alberta (C656) and believed (19) to be part of the indigenous flora. The surprising results of this study are reported in Chapter III.

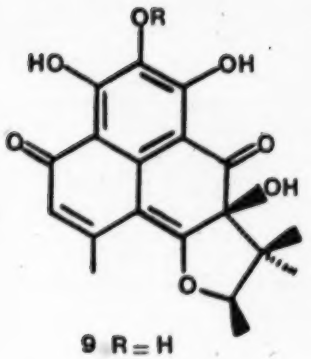
The metabolites isolated from G. abietina either contain a phenalenone (**8**) nucleus fused to a dihydrofuran



8

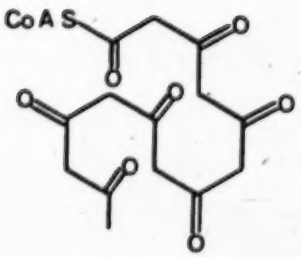
ring, or are presumed to have been derived from such a nucleus. Metabolites containing the phenalenone nucleus with the attached dihydrofuran ring have been isolated from other fungal sources (20). For example,* atrovenetin (**2**), norherqueinone (**9**), and herqueinone (**10**) are produced by Penicillium herquei and P. atrovenetum. Biosynthetic studies (21) on metabolites of P. herquei have shown that these phenalenones are formed from a single heptaketide chain, folded as in **11**. Some of the metabolites of G. abietina, for example sclerodione (**4**) and sclerodin (**1**),

*For a recent review, see ref. 20.



9 R=H

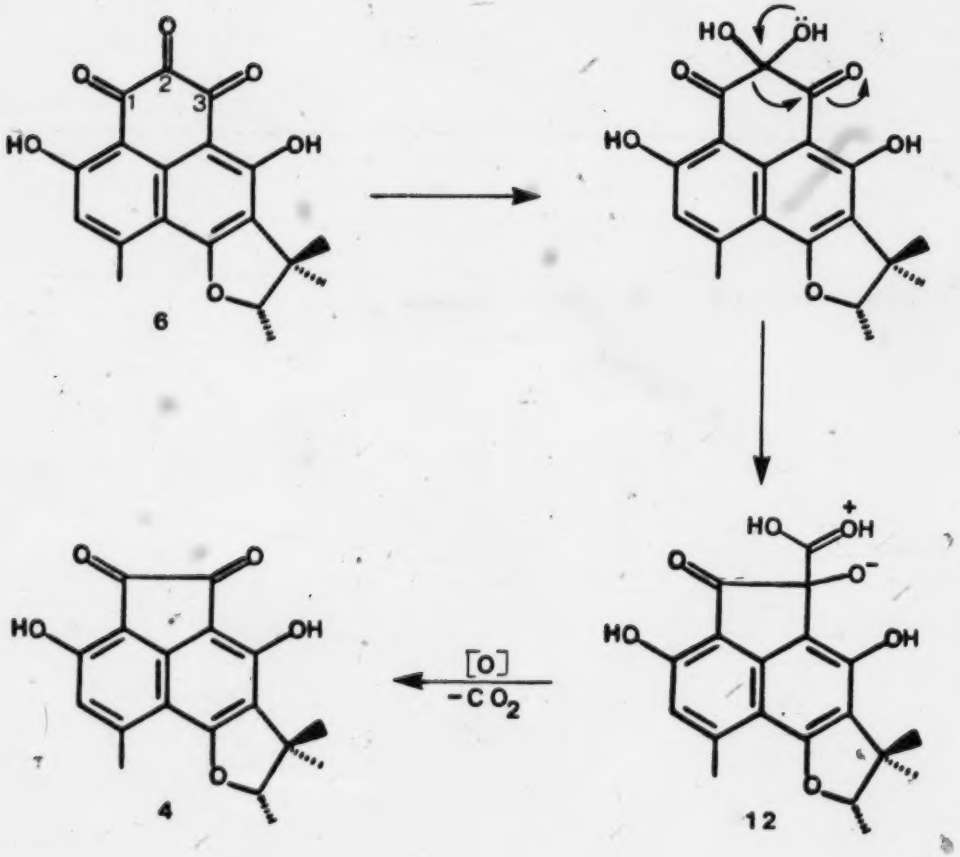
10 R=Me



11

have one carbon missing from the phenalenone nucleus. In principle this degradation could involve the loss of C-1, C-2, or C-3 from, for example, triketone **6** as shown in Scheme I-1. Oxidative decarboxylation of intermediate **12** leads to sclerodione (**4**) which may be a biogenetic precursor of sclerodin (**1**). Biosynthetic studies bearing on these points are described in Chapter IV.

SCHEME I-1



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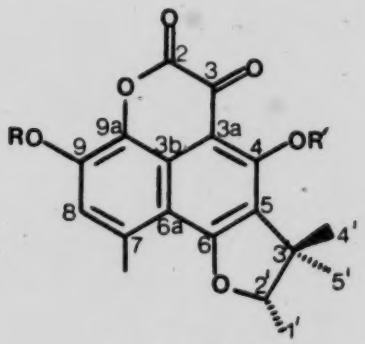
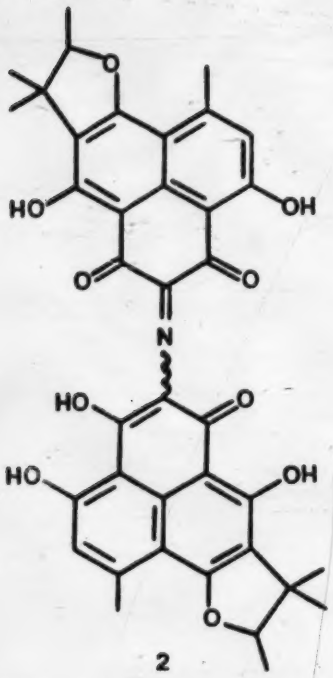
II. METABOLITES PRODUCED BY THE SCLERODERRIS CANKER FUNGUS, GREMMENIELLA ABIETINA. PART 2. THE STRUCTURE OF SCLERODEROLIDE.

In Part 1 of this series (1) we have reported on the isolation of several colored metabolites produced by the Scleroderris canker causing fungus Gremmeniella abietina. One strain of G. abietina (C699) produces a bright yellow metabolite which possesses the biogenetically novel internal phenylglyoxylate lactone structure, 1. In this paper we describe the isolation, structure determination, and some of the chemical properties of this novel compound.



1 R = R' = H
1a R = Ac R' = H
1b R = R' = Ac

Gremmeniella abietina (C699) was grown in still culture, at 16-17°C on a medium of 10% V-8 juice containing 1% added glucose (2). The mycelium was filtered and extracted in a Soxhlet apparatus. Chromatographic separation (silica gel) of the crude metabolites gave Scleroderris blue (2), sclerodin (3),



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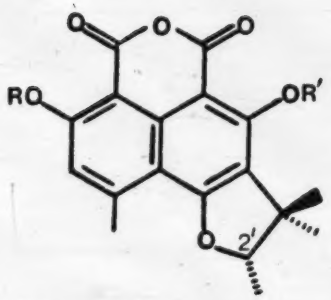
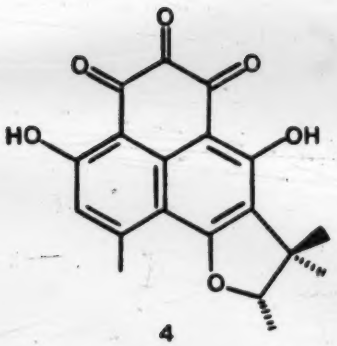


3 R = R′ = H
3a R = Ac R′ = H
3b R = H R′ = Ac
3c R = R′ = Ac
3d R = R′ = Me

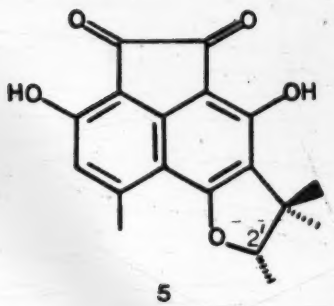
ent-atrovenetinone (4), sclerodione (5), and the yellow metabolite,* for which we propose the name scleroderolide (1).

Scleroderolide (mp 239-240°C) is isomeric with sclerodin (3, $C_{18}H_{16}O_6$) and initially we suspected it to be the isomeric ether 6. The ^1H nmr spectrum of scleroderolide is similar to that of 3 except for the OH

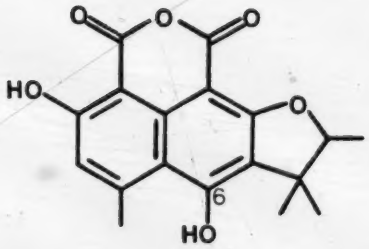
*Isolated for the first time by Y. Hoyano.



4



5



6

hydrogens (δ6.65 and 13.73 vs δ11.43 and 11.64). Scleroderolide readily forms a monoacetate on treatment with acetic anhydride-pyridine, whereas **3** forms the diacetate (1). This is consistent with structure **6**, since an OH group at carbon 6 would be expected to be sterically hindered. However the ^{13}C nmr spectrum of **1** shows

carbonyl absorption at δ170.1 and 155.7, which is not in good agreement with the anhydride structure (δ165.4 and 164.9 in 3). Also the ir spectrum shows carbonyl absorption at 1750 cm^{-1} while that of the anhydride 3 appears at 1705 cm^{-1}.

The monoacetate of scleroderolide crystallizes from dichloromethane-petroleum ether in the form of beautiful red-orange crystals, well suited for an X-ray crystallographic study. Figure II-1 shows a computer generated perspective drawing of the X-ray model of scleroderolide monoacetate, which reveals that it possesses structure **1a**.*

To our surprise, the sample used for X-ray analysis proved to be racemic, though the parent compound (**1**) had an optical rotation of -22.3° (*c* 0.13, $CHCl_3$). Several ~~hypotheses~~ may be entertained to explain these unusual results: 1, scleroderolide was not optically pure (i.e. both enantiomers are produced by *G. abietina*);
2, scleroderolide was racemized during isolation;
3, scleroderolide was racemized during acetylation.
Hypothesis 1 was tested in two ways: using a chiral shift

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*The X-ray structure determination was carried out by J. Clardy and E. Arnold, Department of Chemistry, Cornell University, Ithaca, N.Y. Details are provided in Appendix 1.

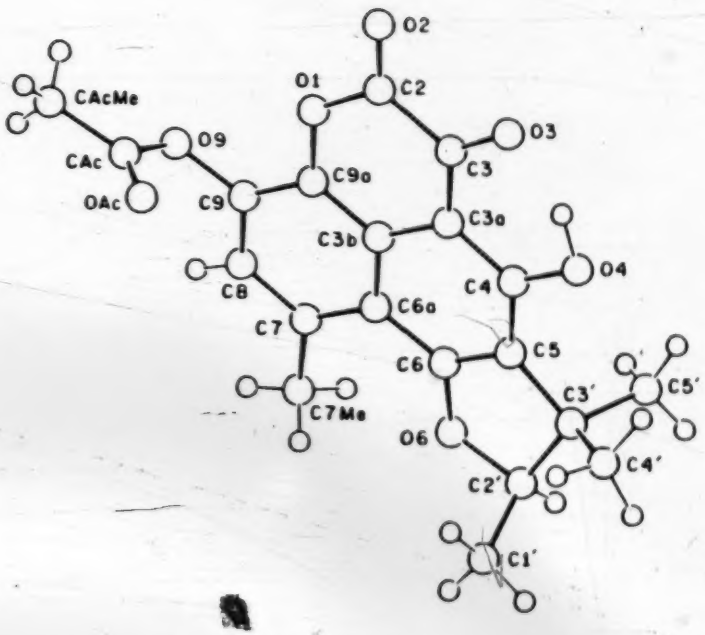
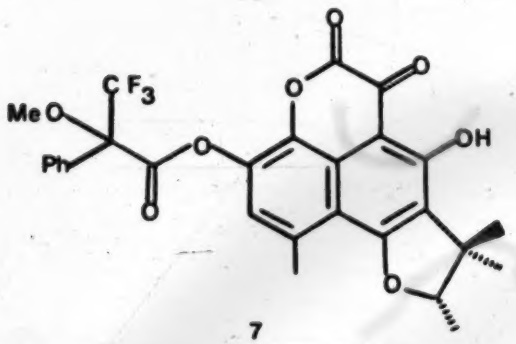


Figure II-1. A computer generated perspective drawing of the X-ray model of scleroderolide monoacetate. The sample used for X-ray analysis was racemic.

reagent (3,4); using an optically active acylating agent.

In the first instance, $Eu(tfc)_3$* was added to scleroderolide (ca. 2 mg in $CDCl_3$) in successively increasing amounts (up to 1 eq.) and the ^1H nmr spectra were obtained; however, we did not see any significant shift but a considerable amount of broadening of the spectral lines was observed. This result is probably due to the lack of effective complexation of the shift reagent with scleroderolide.

We then acylated 1 with the Mosher reagent (5), (-)-α-methoxy-α-trifluoromethyl-α-phenylacetic acid; the ^{19}F nmr spectrum of the resulting monoester 7 (no diester



7

*Tris[3-(trifluoromethylhydroxymethylene)-d-camphorato], europium(III) derivative. Aldrich Chemical Company, Inc.

was formed) showed a single peak at δ-72.17 (internal standard $CFCl_3$). Although we did not observe two ^{19}F signals (indicating two diastereomeric products) this result may be due to the large distance (approximately 9.88 Å) between the two asymmetric centers in **7**.

Sclerodin (**3**) and sclerodione (**5**) were used* to test hypothesis 2. Sclerodione was dissolved in chloroform-trifluoroacetic acid (several ratios) at room temperature (up to 15 days) and at 60°C (1 h); the optical rotation ($[\alpha]_D$ -115.3°) of the recovered material was unchanged. In the case of sclerodin (**3**) we used deuterated solvents (C_6D_6 - CF_3CO_2D) at 80°C (sealed tube) and followed the reaction by ^1H nmr. After 24 h the aromatic hydrogen was exchanged for deuterium; however prolonged treatment (7 days) led to no further changes. The optical rotation ($[\alpha]_D$ -72.6°) of the recovered compound was unchanged. Thus racemization is not likely to have occurred during the isolation of these metabolites.

To test hypothesis 3 scleroderolide was isolated from a fresh culture of G. abietina. Surprisingly this sample of scleroderolide (mp 232-233°C) had a much higher optical rotation ($[\alpha]_D$ -116°) than the previous sample. The

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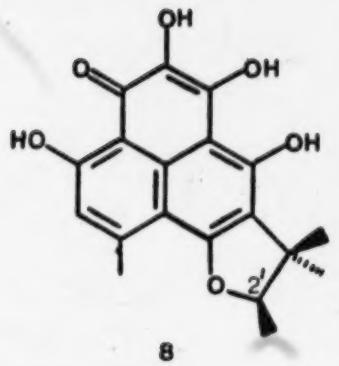
*Scleroderolide is produced by the fungus in smaller amounts and is difficult to purify.

monoacetate from this new preparation showed the same optical rotation ($[\alpha]_D$ -69°)* regardless of its method of formation (acetic anhydride and either pyridine, or 4-N,N-dimethylaminopyridine, or sulfuric acid as catalyst). These results were not due to the selective crystallization of one enantiomer as demonstrated in the following manner: acylation of the combined mother-liquors from recrystallization of scleroderolide (**1**) gave a monoacetate with the same optical rotation as obtained previously. The optical rotation was unchanged after crystallization. These results seem to indicate that G. abietina (C699) produces scleroderolide in high optical purity and that scleroderolide is not racemized during acylation.

Scleroderolide is closely related to atrovenetin, a metabolite of Penicillium atrovenetum (6) and Penicillium herquei (7). The structure of atrovenetin (**8**) was established by X-ray crystallographic analysis (8) and the configuration (R) at C-2' by chemical correlation with (S)-ethyl lactate (9). Interestingly, atrovenetin isolated from P. herquei has a lower optical rotation ($[\alpha]_D$ +54.6°) than atrovenetin isolated from P.

*Scleroderolide isolated as its monoacetate (acetic anhydride-4-N,N-dimethylaminopyridine) from other later cultures showed the same optical rotation.



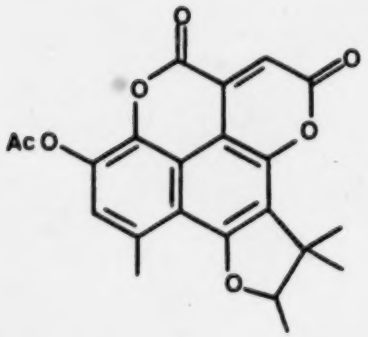
8

atrovenetum ($[\alpha]_D$ +100.6°) (10). Thus in the case of P. herquei, it is possible that both enantiomers of atrovenetin are produced.

In our case, the low optical purity (ca. 20% e.e.) of the initially isolated scleroderolide might be due to a mutant strain, though we cannot exclude completely the possibility that the strain used earlier was misidentified.

Scleroderolide (**1**) is readily converted to the monoacetate **1a** by treatment with acetic anhydride in the presence of pyridine or 4-N,N-dimethylaminopyridine. Utilizing longer reaction times, we attempted to prepare the diacetate **1b**. A major product was obtained, the ^1H nmr spectrum of which was very similar to that of **1a** except that the phenolic OH resonance at δ13.75 was

replaced by a signal at δ6.94 (not D_2O exchangeable). The hrms of this product displayed a molecular ion at m/z 394 ($C_{22}H_{18}O_7$). The ir spectrum shows carbonyl absorption at 1774 and 1738 cm^{-1}. These data are consistent with structure **9**. Compound **9** can result from condensation of **1a** with acetic anhydride followed by cyclization.



9

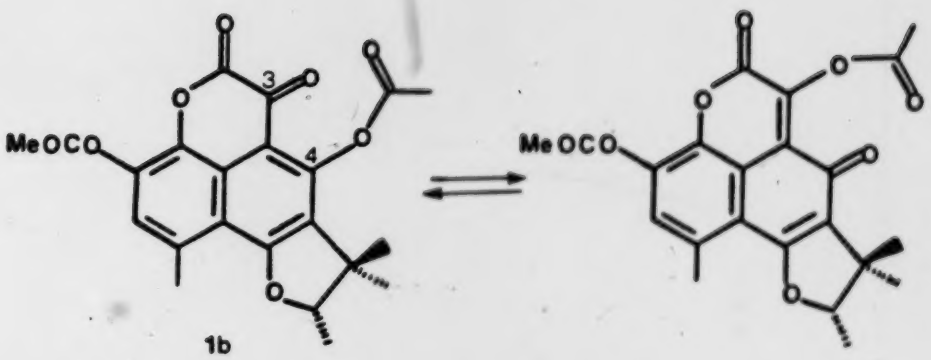
The diacetate **1b** is readily formed under acidic conditions (e.g., acetic anhydride-trifluoroacetic acid). Remarkably, its ^1H and ^{13}C nmr spectra are temperature dependent. For example, the ^1H nmr spectrum of **1b** (in toluene-$\underline{d}_8$) exhibits broad signals for the C-2', C-4' and C-5' hydrogens at room temperature. At 60°C these signals are sharp, whereas at 0°C they appear as resolved pairs. Further cooling to -60°C induces

broadening in the signals for the C-1', C-8, and $ArCH_3$ hydrogens as well as the acetate methyl group at C-9. Interestingly, the acetate methyl at C-4 remains relatively unchanged with temperature. Similarly, the ^{13}C nmr spectrum ($CDCl_3$) shows several broad signals at room temperature, which are sharp at 55°C and are resolved into pairs at -60°C. Since the ^1H and ^{13}C nmr spectra of **1a** do not show this temperature dependence, it appears that the acetate group at C-4 in **1b** is the structural feature responsible for these unusual results. The effect could, in principle, be due to acetyl migration from the C-4 to the C-3 oxygen (Scheme II-1) or to restricted rotation of the acetate group (Scheme II-2). To examine this further, we investigated the nmr spectra of several derivatives of sclerodin (**3**).

Sclerodin diacetate (**3c**) and sclerodin dimethyl ether (**3d**) have previously (1) been prepared. The monoacetate **3a** was obtained from **3** by treatment with acetic anhydride-pyridine, while **3b** may be obtained either from **3** by treatment with acetic anhydride-trifluoroacetic acid (along with the diacetate **3c**) or from **3c** by hydrolysis ($NaHCO_3$).

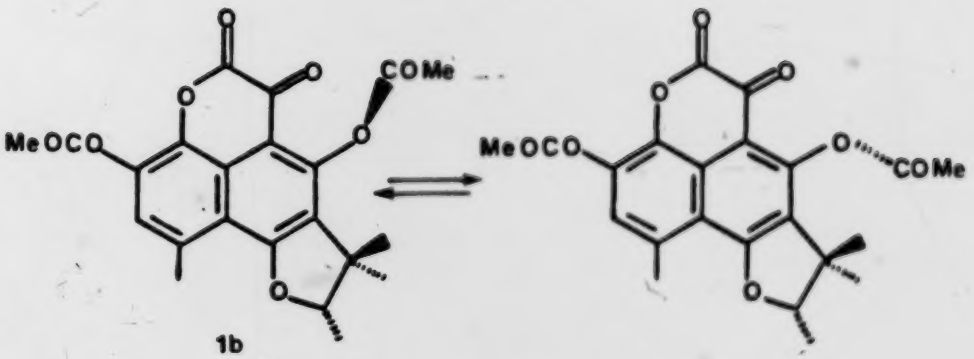
The ^1H nmr spectra of **3d** (1) and **3a** do not show temperature dependence, but the spectra of **3b** and **3c** exhibit temperature dependent features very similar to

SCHEME II-1



1b

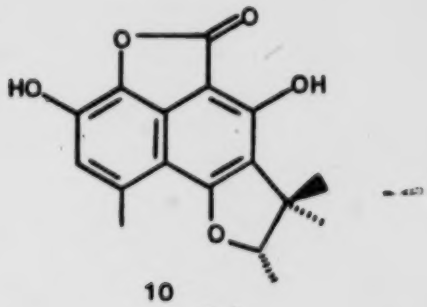
SCHEME II-2



1b

those of **1b**. Since the propensity for acetyl migration is expected to be similar for **3a** and **3b**, we concluded that it is restricted rotation of the acetate group at C-4 which is responsible for the temperature dependent nmr spectra in these compounds.

Sclerodione (**5**), a metabolite of G. abietina (1), on standing in solution is oxidized to sclerodin (**3**); however treatment of 5 with hydrogen peroxide gives 3, along with lactone **10** (1).



10

Reasoning that the lactone **10** may be derived from scleroderolide (**1**) by peroxide oxidation of the corresponding α-keto acid, we decided to investigate the reaction of sclerodione (**5**) with m-chloroperbenzoic acid. To our satisfaction, we found that this reaction led to the formation of both sclerodin (**3**) and scleroderolide (**1**), isolated as its monoacetate **1a**.

The S-configuration at C-2' has been established (1) for some metabolites of G. abietina. The (+)-form ($[\alpha]_D$ +75°) of sclerodin (**3**) has been shown to have the R-configuration at C-2' by X-ray crystallography (11). Consequently sclerodin (**3**, $[\alpha]_D$ -72.6°) from G. abietina is assigned the S-configuration. Ent-atrovenetinone (**4**) and sclerodione (**5**) from G. abietina, on oxidation gave sclerodin (**3**, $[\alpha]_D$ -72.6°) establishing the S-configuration for 4 and 5. Similarly, scleroderolide (**1**, $[\alpha]_D$ -116°) can be prepared, as mentioned above, from sclerodione (**5**), thus allowing the assignment of the S-configuration at C-2' for 1.

To the best of our knowledge, scleroderolide (**1**) is the first natural product possessing the interesting phenylglyoxylate lactone structure. Its biosynthesis will be discussed in Part 4 of this series.

EXPERIMENTAL

All solvents except diethyl ether were distilled prior to use. ACS quality anhydrous diethyl ether was used without purification. Petroleum ether refers to Skelly Oil Company light petroleum (Skellysolve B), bp 62-70°C. Pyridine was distilled from CaH_2 and stored over molecular sieves, acetic anhydride was dried over P_2O_5 and distilled from sodium acetate.

Analytical thin layer chromatography (tlc) was carried out on aluminum sheets (75 × 25 or 75 × 50 mm) pre-coated (0.2 mm) with silica gel $60F_{254}$ (E. Merck, Darmstadt). Preparative thin layer chromatography (ptlc) was carried out on glass plates (20 × 20 cm) pre-coated (0.25 mm) with the same adsorbent (E. Merck, Darmstadt). Materials were detected by visualization under an ultraviolet (uv) lamp (254 or 350 nm), or by spraying with a solution of phosphomolybdic acid (5%) containing a trace of ceric sulfate in sulfuric acid (5%, v/v), followed by charring on a hot plate. Flash column chromatography (12) was performed with Merck Silica Gel 60 (40-63 μm).

High resolution mass spectra (hrms) were recorded on an A.E.I. MS-50 mass spectrometer coupled to a DS 50

computer. Chemical ionization mass spectra (cims)* were recorded on an A.E.I. MS-9 mass spectrometer. Data is reported as m/z (relative intensity). Unless diagnostically significant, peaks with intensities less than 20% of the base peak are omitted. Infrared (ir) spectra were recorded on a Nicolet 7199 FT interferometer. Optical rotations were measured on a Perkin Elmer Model 141 polarimeter. ^{1}H nuclear magnetic resonance (^{1}H nmr) spectra were measured on a Bruker WH-200 spectrometer or a Bruker WH-400 spectrometer. ^{13}C nuclear magnetic resonance (^{13}C nmr) spectra were measured on a Bruker WH-400 spectrometer. For ^{1}H nmr, residual $CHCl_3$ in $CDCl_3$ was employed as the internal standard (assigned as 7.26 ppm downfield from tetramethylsilane (TMS)) and measurements are reported in ppm downfield from TMS (δ). For ^{13}C nmr, $CDCl_3$ was employed as the internal standard (assigned as 77.0 ppm downfield from TMS) and measurements are reported in ppm downfield from TMS (δ). Melting points were recorded on a Fisher-Johns melting point apparatus and are uncorrected.

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*Ammonia as reagent gas.

Growth of *Gremmeniella abietina* and extraction of metabolites

Stock cultures of *Gremmeniella abietina* (C699)* were maintained in slant tubes on potato dextrose agar at 4°C. To initiate large scale cultures, small fragments of agar containing the mycelium were aseptically transferred to Erlenmeyer flasks (5 × 300 mL) containing a sterile culture medium (200 mL) composed of 10% (v/v) clarified V-8** juice and 1% (w/v) glucose. The cultures were shaken for 10 days at 15-17°C. The content of each inoculation flask was transferred to two 2.8 L Fernbach flasks containing the same liquid medium (900 mL) and the flasks (ten) were kept at 15-17°C. After 4 weeks the mycelium was separated from the broth by filtration through cheesecloth. The wet mycelium was extracted with ethyl ether or dichloromethane (24 h), and twice with methanol (24 h), in a Soxhlet extractor. The ether (or dichloromethane) and methanol extracts were dried over sodium sulfate and the solvents evaporated *in vacuo* at 30°C.

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*Obtained from the Northern Forest Research Center, Canadian Forestry Service, Edmonton, Alberta.

**Clarified V-8 juice refers to V-8 (eight garden vegetables, Campbell Soup Company Ltd.) juice filtered through a pad of celite.

Isolation of scleroderolide (1)

Pure scleroderolide (**1**) was obtained from crude mycelium extracts from G. abietima C699. The dichloromethane extract (700 mg) was fractionated by flash column chromatography (dichloromethane-methanol-acetic acid, 47:2:1, 2 L) and the fractions (100 mL) coevaporated with toluene. Fractions 5-7 were refractionated using the same process. After evaporation of the solvent the residue was dissolved in dichloromethane and petroleum ether was added to the solution (brownish) until precipitation occurred. The precipitate was filtered and crystallized from ethanol-petroleum ether to give scleroderolide (**1**, mp 232-233°C); tlc: R_f 0.26 (dichloromethane-petroleum ether-acetic acid, 10:10:1, double elution), yellow spot; $[\alpha]_D$ -116.0° (c 0.30, $CHCl_3$); ir ($CHCl_3$, solution): 3500 (br), 2920, 1750, 1610, 1470, 1380, 1365, 1205, 1030 cm^{-1}; ^1H nmr ($CDCl_3$): δ1.34 (3H, s, C-3' CH_3), 1.51 (3H, d, 6.5 Hz, C-2' CH_3), 1.56 (3H, s, C-3' CH_3), 2.75 (3H, s, $ArCH_3$), 4.77 (1H, q, 6.5 Hz, C-2' H), 6.65 (1H, brs, OH), 6.92 (1H, s, C-8 H), 13.73 (1H, s, OH); ^{13}C nmr ($CDCl_3$)*: δ14.6 (C-1'), 20.6 (C-5'), 22.3 ($ArCH_3$), 25.6

*Details of the assignment are reported in Part 4 of this series.

(C-4'), 43.1 (C-3'), 92.8 (C-2'), 107.2 (C-3a), 109.1 (C-6a), 117.1 (C-8), 119.4 (C-5), 122.2 (C-3b), 129.9 (C-9a), 137.1 (C-7), 144.5 (C-9), 155.7 (C-2), 167.4 (C-4), 169.6 (C-6), 170.1 (C-3); hrms: m/z calcd. for $C_{18}H_{16}O_6$ (M^+): 328.0947; found: 328.0950(50), 300(61), 285(100), 270(9), 267(11), 257(14); cims: m/z 346 (M+18 for 328, 46), 329 (M+1, 100).

Scleroderolide monoacetate (**1a**)

Pure scleroderolide (**1**, 10 mg) was dissolved in dichloromethane (5 mL) and acetic anhydride (excess) and 4-N,N-dimethylaminopyridine were added at room temperature. After 30 min the reaction mixture was diluted with dichloromethane (20 mL) and washed successively with 5% aqueous hydrochloric acid (10 mL), and water (10 mL). The dichloromethane layer was dried (Na_2SO_4), filtered, and concentrated to give the crude product (13 mg). Crystallization from dichloromethane-petroleum ether gave scleroderolide monoacetate (**1a**, mp 183-186°C, 85% yield); tlc: R_f 0.32 (benzene-acetone-acetic acid, 90:10:0.1), yellow spot; $[\alpha]_D$ -69.3° (c 0.28, $CHCl_3$); ir ($CHCl_3$, solution): 3500 (br), 3000, 1760, 1620, 1590, 1470, 1420, 1370, 1300, 1225, 1195, 1030 cm^{-1}; ^1H nmr ($CDCl_3$): δ1.35 (3H, s, C-3' CH_3), 1.52 (3H, d, 6.5 Hz, C-2' CH_3), 1.57 (3H, s, C-3' CH_3), 2.43 (3H, s,

$OOCCH_3$), 2.79 (3H, s, $ArCH_3$), 4.80 (1H, q, 6.5 Hz, C-2' H), 7.01 (1H, s, C-8 H), 13.77 (1H, s, OH); ^{13}C nmr ($CDCl_3$)*: δ14.6 (C-1'), 20.5 (C-5'), 20.6 ($OO\underline{C}CH_3$), 22.3 ($ArCH_3$), 25.6 (C-4'), 43.4 (C-3'), 93.0 (C-2'), 107.6 (C-3a), 113.5 (C-6a), 121.1 (C-5), 122.2 (C-3b), 122.3 (C-8), 135.0 (C-9a), 135.8 (C-7), 137.9 (C-9), 155.1 (C-2), 167.8 (C-4), 168.2 ($OO\underline{C}CH_3$), 169.0 (C-6), 170.7 (C-3); hrms: m/z calcd. for $C_{20}H_{18}O_7$ (M^+): 370.1052; found: 370.1050(26), 328(64), 300(100), 285(96).

Scleroderolide diacetate (**1b**)

Pure scleroderolide (**1**, 10 mg) was dissolved in dichloromethane (5 mL) and acetic anhydride (excess) and trifluoroacetic acid (0.5 mL) were added at room temperature. After 24 hours the reaction mixture was diluted with toluene and concentrated to give the crude product (14 mg). Crystallization from dichloromethane-petroleum ether gave pure scleroderolide diacetate (**1b**, mp 200-202°C, 80% yield); tlc: R_f 0.22 (benzene-acetone-acetic acid, 90:10:0.1), yellow spot; $[\alpha]_D$ -44.6° (*c* 0.28, $CHCl_3$); ir ($CHCl_3$, cast): 2970, 1760, 1670, 1625, 1580, 1530, 1460, 1410, 1360, 1180, 1040 cm^{-1}; ^1H nmr

*For details, see Part 4.

(toluene-$\underline{d}_8$) at 60°C: δ1.0 (3H, s, C-3' CH_3), 1.06 (3H, d, 6.5 Hz, C-2' CH_3), 1.17 (3H, s, C-3' CH_3), 1.82 (3H, s, $OOCCH_3$), 2.20 (3H, s, $OOCCH_3$), 2.60 (3H, s, $ArCH_3$), 4.21 (1H, q, 6.5 Hz, C-2' H), 6.78 (1H, s, C-8 H); ^{13}C nmr ($CDCl_3$) at 55°C: δ14.3 (br)*, 20.5, 21.0, 21.5, 22.4, 25.7 (br)*, 44.0, 92.4, 112.7, 115.9, 123.8, 124.8, 127.9, 134.7, 135.4, 137.6, 152.8, 153.4, 166.8, 168.0, 168.2, 176.2; hrms: m/z calcd. for $C_{22}H_{20}O_8$ (M^+): 412.1158; found: 412.1164(6), 384(7), 370(6), 342(21), 328(12), 300(100), 285(50).

Sclerodin monoacetate (**3a**)

Sclerodin (**3**, 3 mg) was dissolved in dichloromethane and acetic anhydride (excess) and 4-N,N-dimethylamino-pyridine (catalytic amount) were added at room temperature. After 30 min the reaction mixture was diluted with dichloromethane, washed successively with 5% aqueous hydrochloric acid and water. The dichloromethane layer was dried (Na_2SO_4), filtered, and concentrated. Preparative thin layer chromatography (benzene-acetone, 93:7) gave 3a (3 mg); tlc: R_f 0.44 (benzene-acetone, 95:5), blue fluorescence under uv light; ir ($CHCl_3$,

*These signals are sharp at 75°C in toluene-$\underline{d}_8$.

cast): 2980, 1780, 1750, 1670, 1610, 1420, 1290, 1180, 1015 cm^{-1}; ^1H nmr ($CDCl_3$): δ1.28 (3H, s, C-3' CH_3), 1.49 (3H, d, 6.5 Hz, C-2' CH_3), 1.53 (3H, s, C-3' CH_3), 2.42 (3H, s, $OOCCH_3$), 2.86 (3H, s, $ArCH_3$), 4.73 (1H, q, 6.5 Hz, C-2' H), 6.97 (1H, s, C-8 H), 12.14 (1H, s, OH); hrms: m/z calcd. for $C_{20}H_{18}O_7$ (M^+: 370.1052; found: 370.1045(9), 328(37), 313(100), 285(6), 269(6).

Sclerodin monoacetate (**3b**) and sclerodin diacetate (**3c**)

Sclerodin (**3**, 10 mg) was dissolved in dichloromethane and acetic anhydride (excess) and trifluoroacetic acid (0.5 mL) were added at room temperature. After 12 h the reaction mixture was diluted with dichloromethane and washed with water. The dichloromethane layer was dried (Na_2SO_4), filtered and coevaporated with toluene. Preparative thin layer chromatography (benzene-acetone, 95:7) gave **3b** (2 mg) and **3c** (8 mg). Monoacetate **3b** was also prepared by hydrolysis ($NaHCO_3$, H_2O, MeOH; rt; 6 h) of diacetate **3c**.

Sclerodin monoacetate (**3b**); tlc: R_f 0.35 (benzene-acetone, 95:5), blue fluorescence under uv light; ir ($CHCl_3$, cast): 2980, 1780, 1750, 1670, 1610, 1300, 1180, 1030, 860 cm^{-1}; ^1H nmr ($CDCl_3$) at 55°C: δ1.26 (3H, br s, C-3' CH_3), 1.45 (3H, br s, C-3' CH_3), 1.50 (3H, d, 6.5 Hz,

C-2' CH_3), 2.47 (3H, s, $OOCCH_3$), 2.87 (3H, s, $ArCH_3$), 4.69 (1H, q, 6.5 Hz, C-2' H), 6.98 (1H, s, C-8 H), 11.88 (1H, s, OH); ^1H nmr (toluene-$\underline{d}_8$) at 65°: δ1.0 (3H, s, C-3' CH_3), 1.05 (3H, d, 6.5 Hz, C-2' CH_3), 1.16 (3H, s, C-3' CH_3), 2.17 (3H, s, $OOCCH_3$), 2.52 (3H, s, $ArCH_3$), 4.21 (1H, q, 6.5 Hz, C-2' H), 6.56 (1H, s, C-8 H), 12.0 (1H, s, OH); hrms: m/z calcd. for $C_{20}H_{18}O_7$ (M^+): 370.1052; found: 370.1058(12), 328(33), 313(100), 285(5), 269(5).

Sclerodin diacetate (**3c**); tlc: R_f 0.26 (benzene-acetone, 95:5), blue fluorescence under uv light; ir ($CHCl_3$, cast): 2980, 1778, 1760, 1724, 1605, 1600, 1290, 1015 cm^{-1}; ^1H nmr (toluene-$\underline{d}_8$) at 65°C: δ1.0 (3H, s, C-3' CH_3), 1.07 (3H, d, 6.5 Hz, C-2' CH_3), 1.16 (3H, s, C-3' CH_3), 2.13 (3H, s, $OOCCH_3$), 2.16 (3H, s, $OOCCH_3$), 2.55 (3H, s, $ArCH_3$), 4.23 (1H, q, 6.5 Hz, C-2' H), 6.68 (1H, s, C-8 H); hrms: m/z calcd. for $C_{22}H_{20}O_8$ (M^+): 412.1158; found: 412.1161(11), 370(26), 328(64), 313(100).

Scleroderolide α-methoxy-α-trifluoromethyl-α-phenylacetate (**7**)

Pure scleroderolide (**1a**, ca. 3 mg) was dissolved in dichloromethane (1 mL) containing 4-N,N-dimethylamino-

pyridine (excess) and the solution was added to α-methoxy-α-trifluoromethyl-α-phenylacetyl chloride (prepared from the corresponding (-)-acid and oxalyl chloride-dimethylformamide) at room temperature. After 20 min the reaction mixture was diluted with dichloromethane and washed successively with 5% aqueous hydrochloric acid and water. The dichloromethane layer was dried (Na_2SO_4), filtered, and concentrated to give the crude product. Preparative thin layer chromatography (benzene-acetone-acetic acid, 90:10:0.1) gave **7** (2.4 mg); tlc: R_f 0.38 (benzene-acetone-acetic acid, 90:10:0.1), yellow spot; ^1H nmr ($CDCl_3$): δ1.36 (3H, s, C-3' CH_3), 1.53 (3H, d, 6.5 Hz, C-2' CH_3), 1.58 (3H, s, C-3' CH_3), 2.80 (3H, s, $ArCH_3$), 3.81 (3H, s, OCH_3), 4.81 (1H, q, 6.5 Hz, C-2' H), 6.95 (1H, s, C-8 H), 7.53 (3H, m, Ph H's), 7.80 (2H, m, Ph H's), 13.71 (1H, s, OH); hrms: m/z calcd. for $C_{28}H_{23}F_3O_8$ (M^+): 544.1345; found: 544.1348(6), 342(6), 314(7), 189(100).

Anhydroscleroderolide diacetate (**9**)

Pure scleroderolide (**1**, 6 mg) was dissolved in dichloromethane (4 mL) and acetic anhydride (excess) and 4-N,N-dimethylaminopyridine (excess) were added at room temperature. After 2 h the reaction mixture was diluted with dichloromethane and washed successively with 5%

aqueous hydrochloric acid (10 mL) and water (10 mL). The dichloromethane layer was dried (Na_2SO_4), filtered, and concentrated. Preparative thin layer chromatography (benzene-acetone-acetic acid, 90:10:0.1, double elution) gave **9** (45% yield); tlc: R_f 0.37 (benzene-acetone-acetic acid, 90:10:0.1), yellow spot; $[\alpha]_D$ -21.2° (c 0.17, $CHCl_3$); ir ($CHCl_3$, cast): 2980, 1774, 1738, 1590, 1570, 1188, 1032 cm^{-1}; ^1H nmr ($CDCl_3$): δ1.39 (3H, s, C-3' CH_3), 1.52 (3H, d, 6.5 Hz, C-2' CH_3), 1.65 (3H, s, C-3' CH_3), 2.44 (3H, s, $OOCCH_3$), 2.84 (3H, s, $ArCH_3$), 4.76 (1H, q, 6.5 Hz, C-2' H), 6.94 (1H, s, vinylic H), 7.09 (1H, s, C-8 H); hrms: m/z calcd. for $C_{22}H_{18}O_7$ (M^+): 394.1052; found: 394.1050(18), 352(100), 337(67), 300(20); cims: m/z 412 (M+18 for 394, 14), 394(100).

Preparation of scleroderolide (**1**) from sclerodione (**5**)

Sclerodione (**5**, 4.3 mg) was dissolved in dichloromethane (3 mL) and m-chloroperbenzoic acid (3.4 mg) in dichloromethane (1 mL) was added at room temperature. After 5 h the reaction mixture was diluted with dichloromethane and washed with aqueous sodium bicarbonate (sat.). The dichloromethane layer was dried and concentrated to give a mixture of sclerodin (**3**) and scleroderolide (**1**). The aqueous layer was extracted several times with dichloromethane; the dichloromethane extracts were combined, and

evaporated to dryness. tlc of the residue showed a major yellow spot corresponding to scleroderolide. Acetylation (acetic anhydride-4-N,N-dimethylaminopyridine) of this residue gave, after work-up (vide supra), scleroderolide monoacetate **1a**, (2 mg, 40% yield from **5**) identical with an authentic sample (tlc, ir, ^{1}H nmr, and hrms).

Aerial oxidation of sclerodione (**5**)

Sclerodione (**5**, 5 mg) was dissolved in dichloromethane (5 mL) in a loosely stoppered flask. After 5 weeks separation of the reaction mixture by ptlc (dichloromethane-methanol, 49:1, double elution), gave sclerodin (**3**, 1 mg) and recovered sclerodione (3 mg). Sclerodin (**3**) was identical with an authentic sample (tlc, ir, ^{1}H nmr, and hrms).

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III. METABOLITES PRODUCED BY THE SCLERODERRIS CANKER FUNGUS, *GREMMENIELLA ABIETINA*. PART 3. SOME FURTHER METABOLITES.

As part of our study of the metabolites produced by the Scleroderris canker fungus, Gremmeniella abietina, the crude extracts of several strains of the fungus available from the Northern Forest Research Centre (strains C656, C659, C699, C704, C706, C707, and C708) were compared by high pressure liquid chromatography (hplc) in an attempt to correlate the metabolites with the pathogenicity of particular strains. While strain C659 had a very similar chromatographic profile to that of strain C699, the chromatographic profile of strain C656 (and C707) was quite different.*

This report describes the metabolites of G. abietina strain C656 (Alberta) as well as the isolation of a new metabolite from strain C699 (European, from New Brunswick).

Each strain of G. abietina was grown in still culture at 16-17°C on a medium of 10% V-8 juice containing 1% added glucose (2). After 4 weeks the mycelium was harvested and extracted in a Soxhlet apparatus.

The mycelium extract of strain C656 provided a brownish oily material, which was fractionated by silica gel chromatography to give, in order of elution, fatty

*For details, see Appendix 2.

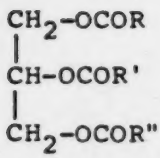
acid methyl esters, sterol esters, triglycerides, 1,3-diglycerides, 1,2-diglycerides, and fatty acids.

The most polar fraction was readily identified as a mixture of fatty acids by its ir and ^1H nmr spectra. The hrms of the methylated (ethereal diazomethane) mixture revealed molecular ions corresponding to stearic ($C_{19}H_{38}O_2$), oleic ($C_{19}H_{36}O_2$), linoleic ($C_{19}H_{34}O_2$) and palmitic ($C_{17}H_{34}O_2$) acid methyl esters. The relative amount of each acid was estimated by integration of the ^1H nmr spectrum (3) as follows: the resonance at δ2.77 is due to the two doubly allylic hydrogens (C-11 H's) of linoleic acid. Since it integrates for 0.5 units and the total methyl groups (δ0.89) for 3.0 units, this acid constitutes approximately 25% (3/2 × 0.5/3.0 × 100) of the mixture. The resonance at δ5.30 (CH=CH), due to oleic and linoleic acids, integrates for 2.0 units, indicating that oleic acid comprises about 50% (3/2 × (2.0-1.0)/3.0 × 100) of the mixture. The remaining 25% is a mixture of stearic and palmitic acids.

The least polar fraction was similarly identified as a mixture of methyl linoleate (ca. 25%), methyl oleate (ca. 40%), methyl stearate and methyl palmitate (ca. 35%).

The fractions of intermediate polarity contained di- and triglycerides, which were identified by means of their ^1H nmr, hrms and cims. The ^1H nmr spectrum of the

triglyceride fraction shows the characteristic signals of a triacylglycerol (3): δ4.20 (2H, dd, 6, 12 Hz), 4.30 (2H, dd, 4.5, 12 Hz), and 5.36 (m, C-2 H) corresponding to five hydrogens of the glyceryl moiety, as well as the resonances due to the fatty acid residues (the signal at δ5.36 overlaps with those of the olefinic protons). Although the hrms of this fraction did not reveal a molecular ion, analysis of the observed fragmentation pattern (4) provided evidence for the fatty acid composition of the triglycerides. Five major tetraoxygenated ion peaks, which represent the loss of an acyloxy group ($[M-RCOO]^+$ or $[M-RCOOH]^+$) were observed: m/z 604 ($C_{39}H_{72}O_4$), 603 ($C_{39}H_{71}O_4$), 602 ($C_{39}H_{70}O_4$), 600 ($C_{39}H_{68}O_4$), and 577 ($C_{37}H_{70}O_4$). These peaks indicate the presence of at least four different fatty acid residues in the triglyceride molecules. The acyl ions ($[RCO]^+$ or $[RCOH]^+$) detected (m/z 265 ($C_{18}H_{33}O$), 264 ($C_{18}H_{32}O$), 263 ($C_{18}H_{31}O$), 262 ($C_{18}H_{30}O$), 239 ($C_{16}H_{31}O$)) indicate the presence of oleic, linoleic, linolenic and palmitic acid residues. Thus this material is a mixture of triglycerides, which may be represented by the general structural formulae:



R, R', R" = $C_{17}H_{35}$, $C_{17}H_{33}$, $C_{17}H_{31}$, $C_{17}H_{29}$, $C_{15}H_{31}$

The 1,3-diglycerides can be distinguished from the 1,2-diglycerides by ^1H nmr (3). The ^1H nmr spectrum of the 1,3-diglycerides shows all of the glyceryl resonances in one multiplet (δ4.18), whereas the ^1H nmr spectrum of 1,2-diglycerides shows four resonances (δ3.76, 4.25, 4.35, and 5.10) for the glyceryl moiety. The cims of the 1,3-diglyceride fraction revealed it to be a complex mixture: m/z (relative intensity) 640(16), 638(72), 636(87), 634(51), 612(73), 610(79). These results indicate the presence of at least four different acyl residues corresponding* to stearic, oleic, linoleic, linolenic, and palmitic acids. The general structural formulae of the components of the 1,3-diglyceride fraction may be represented as follows:

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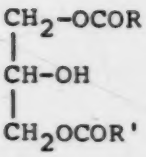
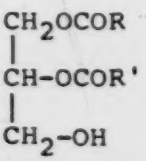
*e.g. 640-18 (NH_4^+) composed of 58 (C_3H_6O) + 283 ($C_{18}H_{35}O_2$) + 281 ($C_{18}H_{33}O_2$).



R, R' = $C_{17}H_{35}$, $C_{17}H_{33}$, $C_{17}H_{31}$, $C_{17}H_{29}$, $C_{15}H_{31}$

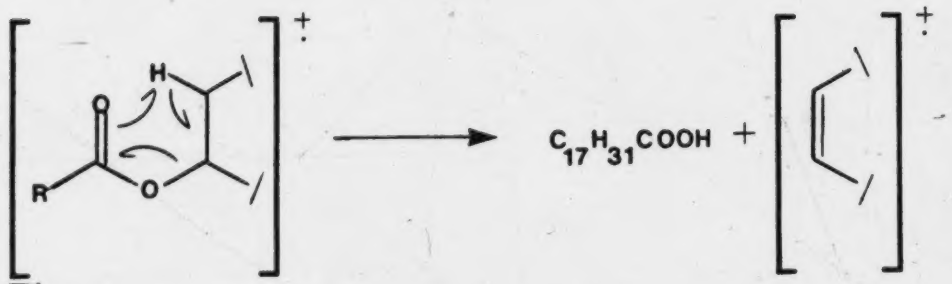
Similar results were obtained with the 1,2-diglycerides. The cims clearly showed that this fraction was a mixture: m/z (relative intensity) 640(13), 638(16), 612(88), 610(18). As above the presence of stearic, oleic, linoleic, linolenic, and palmitic acid residues is consistent with these results. The general structural formulae of the components of the 1,2-diglyceride fraction may be represented as follows:



R, R' = $C_{17}H_{35}$, $C_{17}H_{33}$, $C_{17}H_{31}$, $C_{17}H_{29}$, $C_{15}H_{31}$

Finally, the least abundant of the metabolites produced by strain C656 was tentatively identified as a mixture of three sterol esters. The hrms of this fraction indicated two molecular ions: m/z 662 ($C_{46}H_{78}O_2$) and 660 ($C_{46}H_{76}O_2$) (confirmed by cims). The base peak at m/z 380 ($C_{28}H_{44}$) and a peak at m/z 382 ($C_{28}H_{46}$) suggested the loss

of the same fragment ($C_{18}H_{32}O_2$) from the two molecular ions. This fragment is consistent with loss of linoleic acid (5):



m/z 660 or 662 m/z 382 or 380

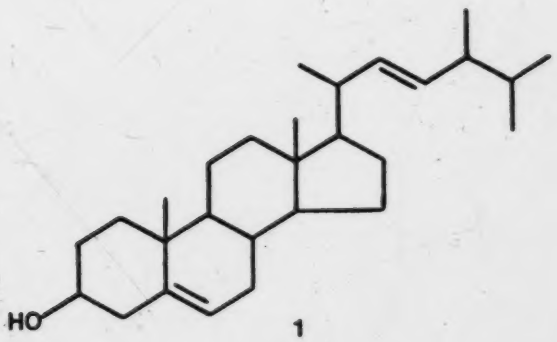
The ^1H nmr spectrum of this material is complex. It shows all the resonances attributable to a doubly unsaturated fatty acid (e.g. linoleic acid), as well as the following signals: δ0.55 (s), 0.60 (s), 0.70 (s), 0.84 (vt), 0.92 (d), 0.98 (s), 4.64 (m), 5.2 (m). The ir shows carbonyl absorption at 1736 cm^{-1}.

The hrms of the neutral portion of the hydrolyzed (6) mixture (methanolic potassium hydroxide) showed a prominent peak at m/z 398 ($C_{28}H_{46}O$). A detailed analysis of the fragmentation pattern is precluded by the complexity of the spectrum. However the major fragments support the hypothesis that it is a C_{28} sterol (6-8): m/z

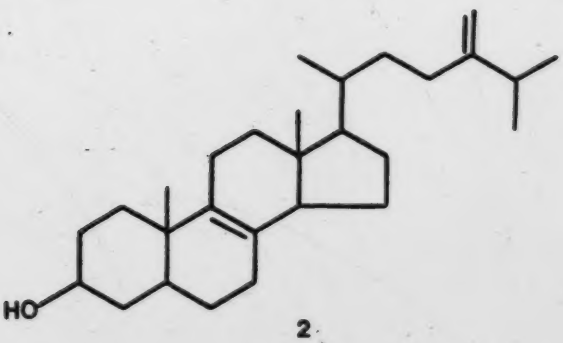
383 (M-CH_3), 300 (M-C_7H_{14}), 271 (M-side chain-2H), 255 (M-side chain-H_2O), 213 (M-side chain-H_2O-42). The ^1H nmr spectrum of this hydrolyzed material is also consistent with a sterol mixture (9). It shows resonances at δ0.54 (s), 0.61 (s), 0.70 (ca. 3H, s), 0.83 (ca. 6H, ap t, 6.5 Hz), 0.90 (ca. 3H, d, 6.5 Hz), 0.94 (s), 0.98-1.02 (m), 3.54 (m), 3.62 (m), 4.68 (<1H, br s); 4.72 (<1H, br s), 5.20 (ca. 2H, m), 5.37 (ca. 1H, m). On acetylation (10) (acetic anhydride-pyridine) the resonances at δ3.54 and 3.62 shifted to δ4.62 and 4.70 (acetyl methyls at δ2.03 and 2.04).

A search of the literature revealed a similarity in the ^1H nmr chemical shifts between some 24-methylsterols (C_{28}) and our mixture. The major component in the mixture is a doubly unsaturated sterol: $C_{28}H_{46}O$, olefinic hydrogens at δ5.20 (m) and 5.37 (m). The C-18 and C-19 hydrogens of $\Delta^{7,22}$ sterols are shifted upfield in relation to $\Delta^{5,22}$ isomers (e.g. δ0.54 and 0.81 vs δ0.69 and 1.02) (9). Therefore the three singlets at δ0.54, 0.61, and 0.70 (relative intensity 3:5:10) observed in the ^1H nmr spectrum of the mixture could be due to the C-18 hydrogens of three different sterols. The chemical shifts for the major component are consistent with those published (9,11) for ergosta-5,22-diene-3β-ol (1). Although the resonances for the other components cannot be clearly assigned, the

presence of signals at δ4.68 (br s) and 4.72 (br s) (second major component) indicates the presence of a terminal methylene group (12). The second major component (C-18 hydrogens at δ0.61) is consistent with ergosta-8,24(28)diene-3β-ol (**2**) (12). A more definite assignment would require the separation of the components, which was not carried out because of the small quantities available.



1



2

The strains of G. abietina previously studied (1) (C699 and C708) produce metabolites possessing the phenalenone skeleton. We could not detect phenalenone metabolites in strain C656 (Alberta). This raises a question regarding the identification of this strain. It is possible that the western fungus (C656) represents a race different from those which occur in Eastern North America (C699 and C708) (13).

During the isolation of scleroderolide* (**3**) from the mycelium extract of G. abietina strain C699, we observed that the thin layer chromatograms of some of the fractions obtained from column chromatography had a blue component which seemed to increase in concentration with time. This blue component had an R_f identical with Scleroderris blue (**4**). This was unexpected since **4** is the least polar of the metabolites (1) isolated from G. abietina. We thus set about to isolate the compound responsible for this behaviour.

The mycelium methanolic extract from strain C699 was washed with cold dichloromethane and the residue was vacuum dried to afford an amorphous greenish-yellow powder (mp > 350°C). This material, upon standing in solution,

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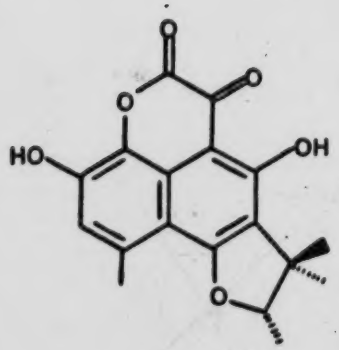
*For details, see Part 2.



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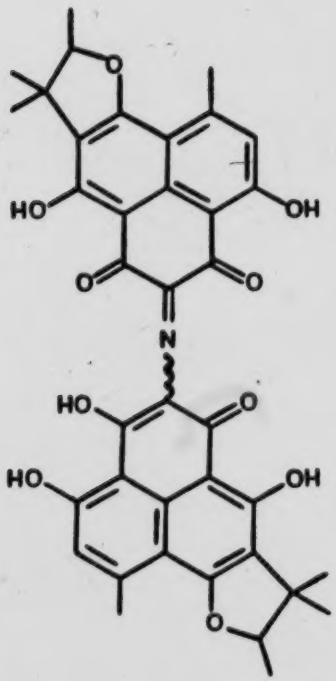
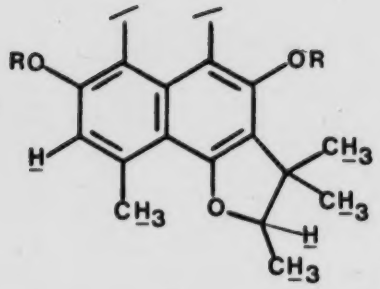


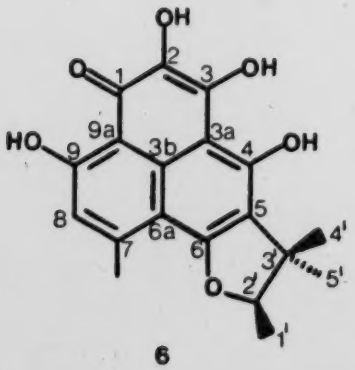
4

was transformed into blue material, which was identical with Scleroderris blue (**4**) by thin layer chromatography comparison. Efforts to crystallize the greenish powder led to decomposition. The ^{1}H nmr spectrum of this material shows resonances consistent with the presence of fragment 5 (underlined hydrogens), as well as four downfield signals (δ 12-15, D_2O exchangeable). The hrms showed a prominent peak at m/z 326 ($C_{19}H_{18}O_5$) and a base peak at m/z 311 ($C_{18}H_{15}O_5$); the cims showed a base peak at m/z 327. These data suggested the molecular formula



5

$C_{19}H_{18}O_5$, which would be consistent with a deoxyatrovenetin structure. Consideration of the biosynthesis of atrovenetin (**6**) (14) led us to formulate structure 7 as a potential deoxyderivative. However, in the ^1H nmr spectrum, we were unable to assign a signal attributable



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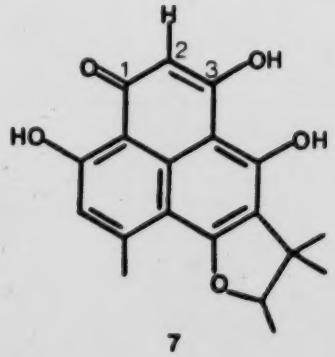
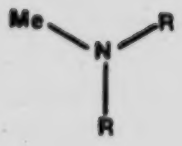


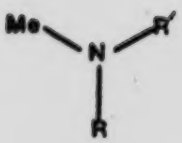
7

to the hydrogen at C-2. A structure such as 7 can exist in several tautomeric forms (the ^{1}H nmr is temperature dependent, indicating a dynamic equilibrium). In order to further study this material and to try at the same time to render it more stable, it was treated with an excess of ethereal diazomethane. After 12 h, tlc of the reaction mixture showed three prominent spots. Preparative tlc (petroleum ether:ethyl acetate, 4:1, multiple elution) of the crude reaction mixture yielded the three components. The hrms of each product indicated the presence of one nitrogen and suggested the molecular formula $C_{43}H_{45}NO_{10}$ (m.w. 735). Analysis of the ^{1}H and ^{13}C nmr spectra allowed the assignment of the "dimeric" structures 8, 9, and 10 to these derivatives.

The ^{1}H nmr spectrum of the most polar component, 8, showed all of the characteristic signals of fragment 5. However, to be consistent with a molecular formula of $C_{43}H_{45}NO_{10}$, the signals must represent twice the number of hydrogens, i.e., it must be a dimeric structure. The remaining protons appear as follows: δ3.68 (3H, s), 3.90 (6H, s), 4.03 (6H, s), 18.50 (2H, s). The diazomethane treatment had apparently led to the introduction of five methyl groups. Except for small differences in chemical shifts, the ^{1}H nmr spectrum of the least polar compound (10) is the same as that for 8.



8



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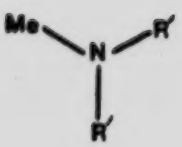
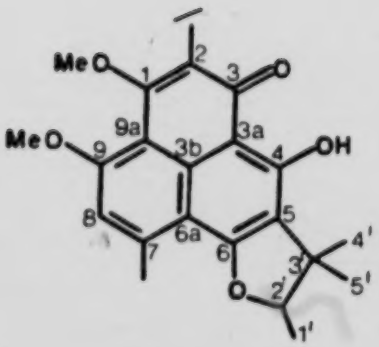


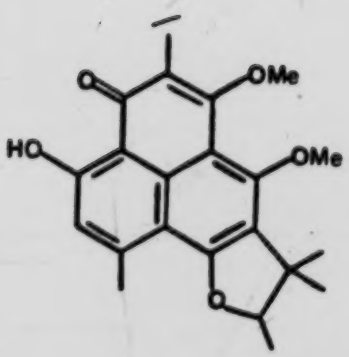
10

R=



11a

R'=



11b

The compound of intermediate polarity (9) has a different ^1H nmr spectrum. Instead of showing signals of double intensity for fragment 5, each signal is paired. The remaining protons are observed as follows: δ3.68 (3H, s), 3.80 (3H, s), 3.87 (3H, s), 3.91 (3H, s), 4.04 (3H, s), 17.91 (1H, s), 18.45 (1H, s). It is noteworthy that

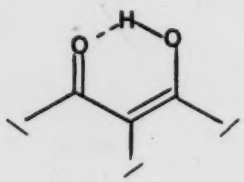
this ^1H nmr spectrum is simulated by superimposing the spectra of compounds 8 and 10.

The ^1H nmr spectra of these three derivatives suggest that two of them have an element of symmetry which renders two fragments of type 5 equivalent, while in the third the symmetry element does not exist. This hypothesis is supported by the ^{13}C nmr spectrum: compound 8 shows 22 signals while compound 9 shows 43 signals.*

From these symmetry considerations several conclusions may be drawn: (1) the nitrogen atom must be involved in linking two identical fragments, (2) the point of attachment in each fragment must be identical, (3) each fragment must contain two OCH_3 groups and the fifth methyl group must be an $N\text{-}CH_3$ group, (4) each fragment must have an internally hydrogen bonded OH group. Thus the structures may be represented as $(C_{21}H_{21}O_5)_2NCH_3$. The fragments $(C_{21}H_{21}O_5)$ must each have two OCH_3 groups, an OH group, and the structural features inherent in 5. These requirements are satisfied by an atrovenetin (6) like structure in which one of the oxygens (at C-1, 2, 3, 4, or 9) is replaced by nitrogen, while two others are methylated. Since there are two different symmetrical

*The ^{13}C nmr of 10 was not obtained because of a lack of sufficient material.

derivatives, as well as an unsymmetrical one, the site of N-substitution must be such as to leave an arrangement of OH groups whereby dimethylation can occur in two ways, leaving two different hydrogen bonded OH groups. Considering the chemical shifts (δ18.50 and 17.91) and the sharpness of the OH signals in the ^{1}H nmr spectra, the following structural feature is a possibility:



Only nitrogen substitution at C-2 can provide a structure in which this feature may appear in two different ways. Thus structures **8**, **9**, and **10** may be proposed for the three derivatives.

The structures were confirmed by a thorough analysis of the ^{13}C nmr spectra. The proton decoupled ^{13}C nmr spectrum of compound **8** shows 22 signals. All except one (δ41.1, NCH_3) of the signals represent a pair of equivalent carbons. The signals may be divided into three groups: 5 sp^2 carbons bound to oxygen (δ150-180); 8 other sp^2 carbons (δ107-150); 9 sp^3 carbons (δ14-91). The sp^3 carbons (C-1', 2', 3', 4', 5', $ArCH_3$, 2×OCH_3, and NCH_3)

and C-8 are readily assigned based on chemical shift and/or multiplicity as well as by analogy to other metabolites (15). The remaining carbons were assigned by analysis of the 2, 3, and 4 bond ^{13}C-^{1}H coupling observed in the fully coupled spectrum (confirmed by selective ^{1}H decoupling) as follows. Since the C-8 hydrogen should have large (ca. 6 Hz) (16) coupling to both C-6a and C-9a and small (ca. 2 Hz) coupling (16) to C-7 and C-9, while only C-6a and C-7 should be coupled to the $ArCH_3$, the assignment of C-6a, C-7, C-9 and C-9a can be made unambiguously. Because of $^{3}J_{CH}$ coupling the assignment of the carbons bearing the OCH_3 and NCH_3 groups is possible by irradiation of the appropriate CH_3 hydrogens (δ 3.68, 3.90, and 4.03). Similarly the carbon bearing the OH group (C-3, 4)* is identified by its $^{2}J_{CH}$ coupling (ca. 3 Hz), while the $^{3}J_{CH}$ coupling serves to locate the α carbons. The C-6 assignment is made on the basis of chemical shift and is confirmed by small (<1 Hz) coupling to the C-2' hydrogen. Of the three remaining carbons (3a, 3b, and 5), C-3b is assigned on the basis of chemical shift as well as its lack of long range coupling. Finally, C-5 is a multiplet due to $^{3}J_{CH}$ coupling to the

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*Because of strong intramolecular hydrogen bonding, C-3 and C-4 are indistinguishable.

C-4' and C-5' hydrogens, while C-3a appears as a doublet if an OH is present at C-4 (or C-3).

The complete ^{13}C nmr assignments for compound **8** are shown in Table III-1. The following observations are pertinent with respect to the proposed structure. Since the resonance at δ160.3 is coupled to both the C-8 hydrogen (δ6.82) and to the lower field OCH_3 hydrogens (δ4.03), a methoxy substituent at C-9 is indicated. Significantly, the OH hydrogen (δ18.50) shows coupling to four resonances: δ107.7 (C-3a), 119.4 (C-5), 175.7, and 177.2. The resonances at δ177.2 and 175.7 are not coupled to OCH_3 hydrogens and therefore must represent a carbonyl carbon and an sp^2 carbon bearing an OH group. This strongly indicates nitrogen substitution at C-2 and thus structure **8**.*

The proton decoupled ^{13}C nmr spectrum of **9** exhibits 43 signals. The presence of fragment **11a** was assumed, since 22 of the signals are identical with the 22 signals observed for **8**, with respect to chemical shift (within 0.2 ppm), multiplicity and long range coupling (confirmed by selective proton decoupling of the NCH_3 (δ3.68), the lower

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*An alternate structure where nitrogen substitution is at C-1 is rigorously ruled out by examination of the ^{13}C nmr spectrum of **9**.

Table III-1. ^{13}C Chemical Shifts[a] of Scleroderris Green Derivatives.

Compound	8	9	
		R	R'
C-1	157.2	157.4	178.2[x]
C-2	133.7	133.6	132.4
C-3	177.2[x]	177.0[x]	156.9[y]
C-3a	107.7	107.6	109.9
C-3b	127.6	127.5	128.0
C-4	175.7[x]	175.9[x]	159.3[y]
C-5	119.3	119.4	126.2
C-6	166.5	166.6	162.2
C-6a	109.3	109.4	108.1
C-7	143.0	143.1	149.2
C-8	110.4	110.4	121.5
C-9	160.5	160.3	176.5[x]
C-9a	110.7	110.5	109.7
$ArCH_3$	23.9	24.0	24.4
C-1'	14.7	14.7	14.4
C-2'	91.1	91.1	90.2
C-3'	43.4	43.4	44.3
C-4'	25.9	25.9	26.2
C-5'	20.5	20.5	22.0
OCH_3	56.4	56.5	61.9
OCH_3	61.3	61.1	64.1
NCH_3	41.1	41.2	

[x,y] Carbon assignments may be interchanged.

[a] In $CDCl_3$.

field OH (δ18.45), the lower field C-8 H (δ6.83), and the lower field $ArCH_3$ (δ2.88)). That the remaining 21 resonances are due to a fragment of structure **11b** was confirmed by analysis of the long range ^{13}C-^{1}H coupling (selective proton decoupling of the NCH_3 (δ3.68), the higher field OH (δ17.91), the higher field C-8 H (δ6.79), and the higher field $ArCH_3$ (δ2.78)), as described above for compound **8**. In this case the OH (δ17.91) is coupled to four resonances: δ109.2 (C-9a), 121.5 (C-8), 176.5 and 178.2. The δ178.2 and 176.5 signals are also coupled to the C-8 hydrogen, thus confirming their assignment as C-1 and C-9. This coupling is uniquely consistent with fragment **11b**. The complete assignment for **9** is given in Table III-1.

The structures **8** and **9** are unambiguously confirmed and that of **10** may be inferred since its ^{1}H nmr spectrum is identical to that of fragment **11b** in **9**.* Thus the structure of the parent compound, for which we suggest the trivial name Scleroderris green, must be **12** (several tautomers possible).

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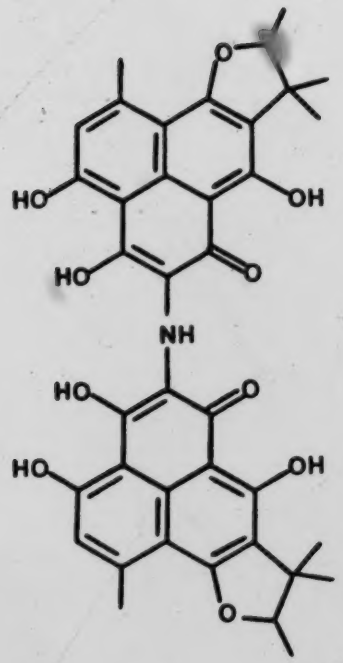
*This is further confirmed by ^{13}C nmr of **10** in which C-2, 3a, 5, 6a, 8, 9a, 1', 4', 5', and $ArCH_3$ are enriched in ^{13}C (see Part 4). The chemical shifts of these carbons are identical (within 0.2 ppm) with the corresponding carbons of **11b** in **9**.



12

Structure 12 is closely related to the structure proposed (1) for Scleroderris blue (**4**). This structural relationship was confirmed as follows. Scleroderris blue was treated with sodium bisulphite (the blue colour gradually changed to violet, green and then yellow). After 18 h the tlc of the crude reaction mixture showed a major spot corresponding to **12**. Treatment of the crude reaction mixture with diazomethane (excess) gave, after

ptlc (petroleum ether-ethyl acetate, 4:1), three products identical with authentic samples of **8** (tlc, ir, ^{1}H nmr, hrms), **9** (tlc, ir, ^{1}H nmr, hrms), and **10** (tlc). In addition, a solution of **12** (dichloromethane-methanol), on standing, gives a blue compound identical (tlc) with Scleroderris blue (**4**). These results serve to confirm the structure proposed for Scleroderris blue (**4**).

The wood of Scleroderris infected pine shows a characteristic yellowish green coloration (17). It is possible that compound **12** causes this discolouration.

The crude extracts of G. abietina C699 show biological activity against several microorganisms.*

*For details, see Appendix 3.

EXPERIMENTAL

All solvents except diethyl ether were distilled prior to use. ACS quality anhydrous diethyl ether was used without purification. Petroleum ether refers to Skelly Oil Company light petroleum, bp 62-72°C. Pyridine was distilled from CaH_2 and stored over molecular sieves, acetic anhydride was dried over P_2O_5 and distilled from sodium acetate.

Analytical thin layer chromatography (tlc) was carried out on aluminum sheets (75 × 25 or 75 × 50 mm) pre-coated (0.2 mm) with silica gel $60F_{254}$ (E. Merck, Darmstadt). Preparative thin layer chromatography (ptlc) was carried out on glass plates (20 × 20 cm) pre-coated (0.25 mm) with the same adsorbent (E. Merck, Darmstadt). Materials were detected by visualization under an ultraviolet (uv) lamp (254 or 350 nm), or by spraying with a solution of phosphomolybdic acid (5%) containing a trace of ceric sulfate in aqueous sulfuric acid (5% v/v), followed by charring on a hot plate. Flash column chromatography (18) was performed with Merck Silica Gel (40-63 μm). High-pressure liquid chromatography (hplc) was carried out with a Waters Associates liquid chromatograph, consisting of two solvent delivery systems, Model M45 and Model 6000A,

an injector, Model WISP 710B, a uv absorbance detector, Model 480, a data handling system, Model 730, a system controller, Model 720, and a radial compression separation system, Z-Module (equipped with a μBONDAPAK C_{18} Radial-PAK Cartridge 8 mm × 10 cm).

High resolution mass spectra (hrms) were recorded on an A.E.I. MS-50 mass spectrometer coupled to a DS 50 computer. Chemical ionization* mass spectra (cims) were recorded on an A.E.I. MS-9 mass spectrometer. Data is reported as m/z (relative intensity). Unless diagnostically significant, peaks with intensities less than 20% of the base peak are omitted. Infrared (ir) spectra were recorded on a Nicolet 7199 FT interferometer. Ultraviolet (uv) spectra were recorded on a Hewlett-Packard HP8450A Diode Array spectrometer coupled to a 7470A plotter. Optical rotations were measured on a Perkin Elmer Model 141 polarimeter. ^1H nuclear magnetic resonance (^1H nmr) spectra were measured on a Bruker WH-200 spectrometer or a Bruker WH-400 spectrometer. ^{13}C nuclear magnetic resonance (^{13}C nmr) spectra were measured on a Bruker WH-400 spectrometer. For ^1H nmr, residual $CHCl_3$ in $CDCl_3$ was employed as the internal standard

*Ammonia as reagent gas.

(assigned as 7.26 ppm downfield from tetramethylsilane (TMS)) and measurements are reported in ppm downfield from TMS (δ). For ^{13}C nmr, $CDCl_3$ was employed as the internal standard (assigned as 77.0 ppm downfield from TMS) and measurements are reported in ppm downfield from TMS (δ). Melting points were recorded on a Fisher-Johns melting point apparatus and are uncorrected.

Growth of Gremmeniella abietina and extraction of metabolites

Gremmeniella abietina (C656, C659, C699, C704, C706, C707 and C708)* was grown in liquid still culture at 16-17°C, as reported previously.** After 4 weeks the mycelium was separated from the broth, washed with cold methanol, and dried. The dry mycelium was extracted with ethyl ether (24 h) and twice with methanol (24 h each) in a Soxhlet apparatus. The ether and methanol extracts were separately concentrated in vacuo at 30°C.

*Obtained from the Northern Forest Research Center, Canadian Forestry Service, Edmonton.

**For details, see Experimental, Part 2 of this series.

Isolation of metabolites from G. abietina C656

The crude mycelium ether extract of G. abietina C656 gave ca. 0.1 g/L of crude metabolites and the combined crude methanol extracts gave 0.4 g/L. Both extracts were brownish oils (similar by tlc).

The crude mycelium ether extract (0.8 g) was fractionated by flash column chromatography with the following solvent system: dichloromethane-methanol (49:1, 1.5 L), dichloromethane-methanol (46:2, 1 L), and methanol (0.5 L). Fractions of 125 mL were collected. Fractions 2-4 and 15 were further purified as indicated for the individual components.

Fatty acids

The fatty acids were eluted in the most polar fractions. Fractions 19-21 (50 mg) gave a single spot on tlc; tlc: R_f 0.16 (dichloromethane-methanol, 49:1); ir ($CHCl_3$, cast): 2925, 2855, 1711, 1460, 1280, 938, 720 cm^{-1}; ^1H nmr ($CDCl_3$): δ0.89 (vt, 6.5 Hz, CH_3), 1.26-1.32 (m, $(CH_2)_n$), 1.64 (m, $C\underline{H}_2CH_2CO$), 2.0-2.08 (m, $C\underline{H}_2CH=$), 2.34 (t, 8.0 Hz, $C\underline{H}_2CO$), 2.77 (t, 6.5 Hz, $=CHC\underline{H}_2CH=$), 5.30-5.42 (m, $C\underline{H}=C\underline{H}$); hrms: m/z 97(21), 95(30), 83(39), 82(26), 81(50), 69(65), 68(29), 67(61), 57(49), 56(25), 55(100); cims: m/z 300 (M+18 for 282, 26), 298 (M+18 for 280, 74), 296 (M+18 for 278, 87).

Methylation of fatty acids: the mixture of fatty acids (5 mg) was dissolved in dichloromethane (1 mL) and treated with excess ethereal diazomethane to give fatty acid methyl esters (5 mg); tlc: R_f 0.60 (petroleum ether-ethyl acetate, 9:1); hrms: m/z 298(25), 296(27), 294(34), 270(13), 264(95), 262(14), 55(100).

Fatty acid methyl esters

The fatty acid methyl esters were eluted in fractions 2-4 (80 mg). Preparative tlc (petroleum ether-ethyl acetate, 19:1, double elution) of these combined fractions gave a mixture of fatty acid methyl esters (10 mg). tlc: R_f 0.60 (petroleum ether:ethyl acetate, 9:1); ir ($CHCl_3$, cast): 3100, 2925, 2854, 1743, 1460, 1430, 1165, 720 cm^{-1}; ^1H nmr ($CDCl_3$): δ0.89 (vt, 6.5 Hz, CH_3), 1.26-1.32 (m, $(CH_2)_n$), 1.64 (m, $\underline{CH_2}CH_2CO$), 2.0-2.08 (m, $\underline{CH_2}CH=$), 2.28 (t, 8.0 Hz, $\underline{CH_2}CO$), 2.75 (t, 6.5 Hz, $=CH\underline{CH_2}CH=$), 3.67 (s, OCH_3), 5.30-5.40 (m, $\underline{CH}=\underline{CH}$); hrms: m/z 298(3), 296(2), 294(2), 270(12), 264(7), 74(100).

Triglycerides

The triglycerides were eluted in fractions 2-4 (80 mg). Preparative tlc (petroleum ether-ethyl acetate, 19:1) of these combined fractions gave a mixture of triglycerides (30 mg); tlc: R_f 0.36 (petroleum ether-ethyl acetate, 9:1); ir ($CHCl_3$, cast): 3010, 2925, 2854,

1745, 1463, 1163, 720 cm^{-1}; ^1H nmr ($CDCl_3$): δ0.89 (vt, 6.5 Hz, CH_3), 1.28-1.32 (m, $(CH_2)_n$), 1.62 (m, $\underline{CH_2}CH_2CO$), 2.05 (m, $\underline{CH_2}CH=$), 2.32 (t, 7.0 Hz, $CH_2\underline{CH_2}CO$), 2.78 (brt, 6.0 Hz, $=CH\underline{CH_2}CH=$), 4.18 (dd, 6.0, 12 Hz, $O\underline{CH_2}CH(O)\underline{CH_2}O$), 4.30 (dd, 4.5, 12 Hz, $O\underline{CH_2}CH(O)\underline{CH_2}O$), 5.36 (m, $\underline{CH}=\underline{CH}$, $OCH_2\underline{CH}(O)CH_2O$); hrms: m/z 604(45), 603(80), 602(63), 601(40), 600(43), 577 (78), 576(39), 574(25), 341(7), 339(28), 337(8), 313(19), 265(24), 264(36), 263(32), 262(89), 239(11), 55(100).

1,3-Diglycerides

The 1,3-diglycerides were eluted in fractions 6-10. Fraction 8 (10 mg) gave a single spot on tlc; tlc: R_f 0.45 (dichloromethane-methanol, 49:1); ir ($CHCl_3$, cast): 3460 (br), 2925, 2854, 1743, 1465, 1170, 720 cm^{-1}; ^1H nmr ($CDCl_3$): δ 0.90 (vt, 6.5 Hz, CH_3), 1.28-1.32 (m, $(CH_2)_n$), 1.62 (m, $\underline{CH_2}CH_2CO$), 2.04 (m, $\underline{CH_2}CH=$), 2.37 (t, 7.0 Hz, $CH_2\underline{CH_2}CO$), 2.48 (d, 4.5 Hz, OH), 2.78 (brt, 6.0 Hz, $=CH\underline{CH_2}CH=$), 4.18 (m, $O\underline{CH_2}\underline{CH}(OH)\underline{CH_2}O$), 5.36 (m, $\underline{CH}=\underline{CH}$); hrms: m/z 602(2), 600(2), 576(2), 339(39), 313(27), 265(23), 264(26), 262(35), 239(15), 55(100); cims: m/z 640 (M+18 for 622, 16), 638 (M+18 for 620, 72), 636 (M+18 for 618, 87), 634 (M+18 for 616, 51), 612 (M+18 for 594, 74), 610 (M+18 for 592, 80).

1,2-Diglycerides

The 1,2-diglycerides were eluted in fractions 12-16 (50 mg). Preparative tlc (dichloromethane-methanol, 97:3) of fraction 15 gave a mixture of 1,2-diglycerides (6 mg); tlc: R_f 0.33 (dichloromethane-methanol, 49:1); ir ($CHCl_3$, cast): 3440 (br), 2925, 2854, 1740, 1463, 1170, 720 cm^{-1}; ^1H nmr ($CDCl_3$): δ0.90 (vt, 6.5 Hz, CH_3), 1.28-1.32 (m, $(CH_2)_n$), 1.63 (m, $C\underline{H}_2CH_2CO$), 2.03-2.08 (m, $C\underline{H}_2CH=$, $O\underline{H}$), 2.34 (m, $CH_2C\underline{H}_2CO$), 2.79 (brt, 6.0 Hz, $=CHC\underline{H}_2CH=$), 3.76 (t, 5.0 Hz, $C\underline{H}_2OH$), 4.25 (dd, 6.0, 12 Hz, $OC\underline{H}_2CH(O)CH_2OH$), 4.35 (dd, 4.5, 12 Hz, $OC\underline{H}_2CH(O)CH_2OH$), 5.10 (m, $OCH_2C\underline{H}(O)CH_2OH$), 5.36 (m, $C\underline{H}=C\underline{H}$); hrms: m/z 604(4), 602(4), 576(19), 341(16), 339(39), 313(75), 265(26), 264(40), 262(16), 239(32), 55(100); cims: m/z 640 (M+18 for 622, 13), 638 (M+18 for 620, 16), 612 (M+18 for 594, 88), 610 (M+18 for 592, 18).

Sterol esters

The sterol esters were eluted in fractions 2-4. Preparative tlc (petroleum ether-ethyl acetate, 97:3, double elution) of these combined fractions gave a mixture of sterol esters (5 mg); tlc: R_f 0.55 (petroleum ether-ethyl acetate, 9:1); ir ($CHCl_3$, cast): 2954, 2928, 1736, 1460, 1175, 720 cm^{-1}; ^1H nmr ($CDCl_3$): see text; hrms: m/z 662(1), 660(2), 535(1), 382(24), 381(42), 380(100),

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337(4), 282(5), 255(36), 253(6); cims: m/z 678 (M+18 for 660, 69), 398(100).

Hydrolysis of sterol esters: the mixture of sterol esters (4 mg), was dissolved in dichloromethane and 10% methanolic potassium hydroxide (excess) was added. After refluxing for 1 h, the reaction mixture was cooled, diluted with dichloromethane, washed with 5% hydrochloric acid, and concentrated. The resulting residue was fractionated by ptlc (petroleum ether-ethyl acetate, 4:1, double elution) to give a mixture of sterols (2 mg); tlc: R_f 0.26 (petroleum ether-ethyl acetate, 4:1); ir ($CHCl_3$, cast): 3400 (br), 2955, 2424, 2853, 1728 (w), 1460, 1370, 1060, 980 cm^{-1}; ^1H nmr ($CDCl_3$): see text; hrms: m/z 400(11), 398(68), 396(14), 383(16), 363(12), 314(12), 300(20), 271(52), 255(28), 69(90), 55(100).

Acetylation of sterols: the sterol mixture (2 mg) was dissolved in dichloromethane (1 mL) and acetic anhydride (excess) and pyridine (excess) were added at room temperature. After 24 h the reaction mixture was diluted with dichloromethane, washed with 5% hydrochloric acid, dried (Na_2SO_4) and concentrated to give a mixture of sterol acetates; tlc: R_f 0.70 (petroleum ether-ethyl acetate, 4:1); ir ($CHCl_3$, cast): 2955, 2860, 1730 (s), 1460, 1375, 1240, 1040, 970 cm^{-1}; ^1H nmr ($CDCl_3$): see text; hrms: m/z 440(16), 398(16), 382(20), 380(100),

313(19), 255(38).

Isolation of Scleroderris green (12)

Scleroderris green (12) was isolated from the second methanol extract from G. abietina C699. The crude mycelial extract was washed several times with cold dichloromethane and the residue was vacuum dried to afford ca. 200 mg of an amorphous greenish-yellow powder (mp > 340°C). Attempts at crystallization (CH_2Cl_2, EtOAc, MeOH, etc.) led to decomposition; tlc: R_f 0.65 (benzene-acetone, 9:1), yellowish spot (with streaking), which turns blue on standing; uv (MeOH) λ_{max}: 214(0.79), 259(0.48), 396(0.29) nm; ir ($CHCl_3$), cast): 3320 (br), 2920, 1617, 1380, 1280, 1060 cm^{-1}; ^1H nmr ($CDCl_3$) at 50°C: δ1.33 (6H, brs, C-3' CH_3), 1.48 (6H, d, 6.6 Hz, C-2' CH_3), 1.56 (6H, brs, C-3' CH_3), 2.84 (6H, s, $ArCH_3$), 4.69 (2H, q, 6.6 Hz, C-2' H), 6.88 (2H, s, C-8 H), 12.44, 13.28, 13.42, 14.26 (ca. 4H, D_2O exchangeable); hrms: m/z 340(15), 326(38), 311(100), 269(24); cims: m/z 327(100).

Methylation of Scleroderris green

Scleroderris green 12 (ca. 40 mg) was suspended in dichloromethane (10 mL) and was treated with excess ethereal diazomethane. After 12 h, the crude reaction mixture was evaporated to dryness and fractionated by ptlc

(petroleum ether-ethyl acetate, 4:1, multiple elution), to give compounds **8** (8 mg), **9** (8 mg), and **10** (3 mg). Compounds **8** (orange crystals, mp 282-283°C) and **9** (red crystals, mp 275-277°C) were crystallized from dichloromethane-methanol.

Compound **8**; tlc: R_f 0.29 (petroleum ether-ethyl acetate, 3:1), yellow spot; $[\alpha]_D$ -36.4° (c 0.44, $CHCl_3$); ir ($CHCl_3$, cast): 3400 (br), 2920, 1607, 1580, 1452, 1354, 1300, 1223 cm^{-1}; ^1H nmr ($CDCl_3$): δ 1.32 (6H, s, C-3' CH_3), 1.46 (6H, d, 6.5 Hz, C-2' CH_3), 1.54 (6H, s, C-3' CH_3), 2.87 (6H, s, $ArCH_3$), 3.68 (3H, s, NCH_3), 3.90 (6H, s, OCH_3), 4.03 (6H, s, OCH_3), 4.64 (2H, q, 6.5 Hz, C-2' H), 6.82 (2H, s, C-8 H), 18.50 (2H, s, OH); ^{13}C nmr ($CDCl_3$): see Table III-1; hrms: m/z calcd. for $C_{43}H_{45}NO_{10}$ (M^+): 735.3043; found: 735.3035(71), 704(100); cims: m/z 736(100).

Compound **9**; tlc: R_f 0.35 (petroleum ether-ethyl acetate, 3:1), orange spot; $[\alpha]_D$ -78.6° (c 0.15, $CHCl_3$); ir ($CHCl_3$, cast): 3400 (br), 2920, 1607, 1580, 1455, 1354, 1299 cm^{-1}; ^1H nmr ($CDCl_3$): δ1.27 (3H, s, C-3' CH_3), 1.33 (3H, s, C-3' CH_3), 1.46 (3H, d, 6.5 Hz, C-2' CH_3), 1.47 (3H, d, 6.5 Hz, C-2' CH_3), 1.52 (3H, s, C-3' CH_3), 1.56 (3H, s,

C-3' CH_3), 2.78 (3H, s, $ArCH_3$), 2.88 (3H, s, $ArCH_3$), 3.68 (3H, s, NCH_3), 3.80 (3H, s, OCH_3), 3.87 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 4.04 (3H, s, OCH_3), 4.55 (1H, q, 6.5 Hz, C-2' H), 4.64 (1H, q, 6.5 Hz, C-2' H), 6.79 (1H, s, C-8 H), 6.83 (1H, s, C-8 H), 17.91 (1H, s, OH), 18.45 (1H, s, OH); ^{13}C nmr ($CDCl_3$): see Table III-1; hrms: m/z calcd. for $C_{43}H_{45}NO_{10}$ (M^+): 735.3043; found: 735.3061(100), 704(80), 366(24), 341(29); cims: m/z 736(100).

Compound **10**; tlc: R_f 0.48 (petroleum ether-ethyl acetate, 3:1), red spot; ir ($CHCl_3$, cast): 3400 (br), 2925, 1609, 1586, 1458, 1318, 1296 cm^{-1}; ^1H nmr ($CDCl_3$): δ1.27 (6H, s, C-3' CH_3), 1.46 (6H, d, 6.5 Hz, C-2' CH_3), 1.52 (6H, s, C-3' CH_3), 2.80 (6H, s, $ArCH_3$), 3.67 (3H, s, NCH_3); 3.75 (6H, s, OCH_3), 3.86 (6H, s, OCH_3), 4.56 (2H, q, 6.5 Hz, C-2' H), 6.82 (2H, s, C-8 H), 17.91 (2H, s, OH); ^{13}C nmr ($CDCl_3$):* δ22.0 (C-5'), 24.3 ($ArCH_3$), 26.2 (C-4'), 90.3 (C-2'), 108.1 (C-6a), 109.7 (C-9a), 109.8 (C-3a), 121.5 (C-8), 126.2 (C-5), 132.4 (C-2); hrms: m/z calcd. for $C_{43}H_{45}NO_{10}$ (M^+): 735.3043; found: 735.3054(100), 704(69), 367(39), 366(44), 341(26); cims: m/z 736(100).

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*All carbons are enriched in ^{13}C.

Reduction of Scleroderris blue (**4**)

Scleroderris blue (**4**, 3 mg) was dissolved in dichloromethane-methanol (1:1, 2 mL) and aqueous sodium bisulfite (ca. 20 mg) was added with stirring at room temperature (the color of the reaction mixture changed from blue to violet, then green, and finally light yellow). After 18 h the reaction mixture was diluted with dichloromethane, washed with water (twice), dried, and concentrated. tlc (benzene-acetone-acetic acid, 90:10:0.1) of the residue showed a major spot corresponding to Scleroderris green (**12**). To the residue (3 mg) in dichloromethane (1 mL) was added ethereal diazomethane (excess) at room temperature. After 12 h the solvents were evaporated and the residue was fractionated by ptlc (petroleum ether-ethyl acetate, 4:1, multiple elution) to give compounds **8** (0.5 mg), **9** (0.5 mg) and **10** (<0.3 mg). Compounds **8** and **9** were identical with authentic samples by tlc, ir, ^{1}H nmr and hrms comparison; compound **10** was identical with an authentic sample by tlc.

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IV. METABOLITES PRODUCED BY THE SCLERODERRIS CANKER FUNGUS, *GREMMENIELLA ABIETINA*. PART 4. BIOSYNTHETIC STUDIES.

We have previously reported (1)* the isolation of several metabolites from Gremmeniella abietina, which contain the phenalenone nucleus fused to a dihydrofuran ring. Several structurally related metabolites have been isolated from other fungi.** Biosynthetic studies on metabolites of Penicillium herquei (e.g. 1) have revealed a heptaketide origin for the phenalenone nucleus, whereas the dihydrofuran ring is mevalonate derived (Scheme IV-1) (3). The metabolites of G. abietina are expected to have a similar biosynthetic origin. Three of the metabolites (3, 4, and 5) have one carbon atom less than the parent phenalenones (e.g. 1 and 2). In principle such a degradation could involve the loss of C-1, C-2, or C-3. We had previously speculated (4) that ent-atrovenetinone (2) could be a precursor of sclerodione (3), by loss of C-2. A possible pathway from 2 to 3 is illustrated in Scheme IV-2. Oxidation of 3 could lead to both sclerodin (5) and scleroderolide (4) (Scheme IV-3). To examine the nature of this degradation we studied the incorporation of [1-^{13}C] and [2-^{13}C] acetate by G. abietina into compounds 3, 4, and 5, as well as into the recently described

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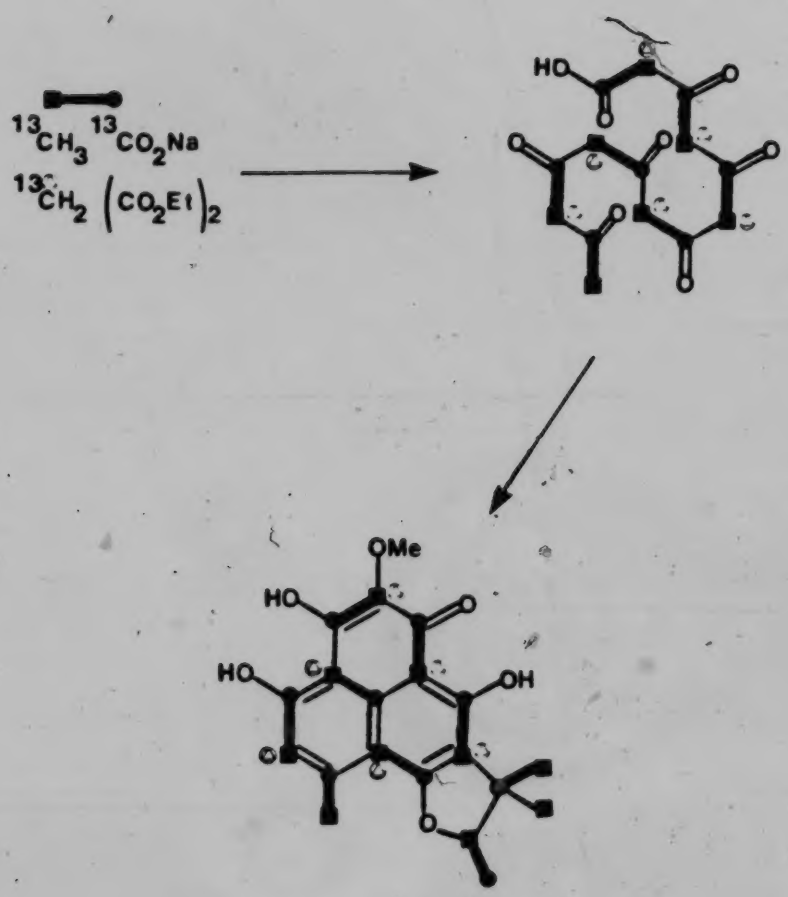
*See also Parts 2 and 3 of this series.

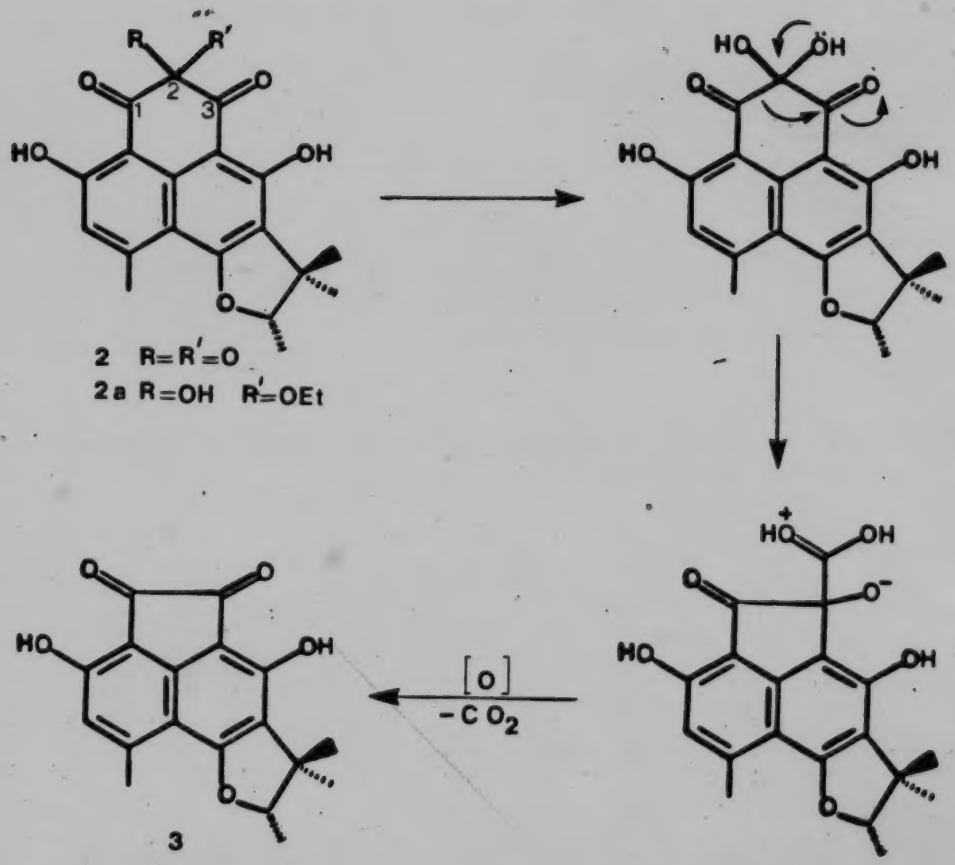
**For a recent review, see ref. 2.

SCHEME IV-1

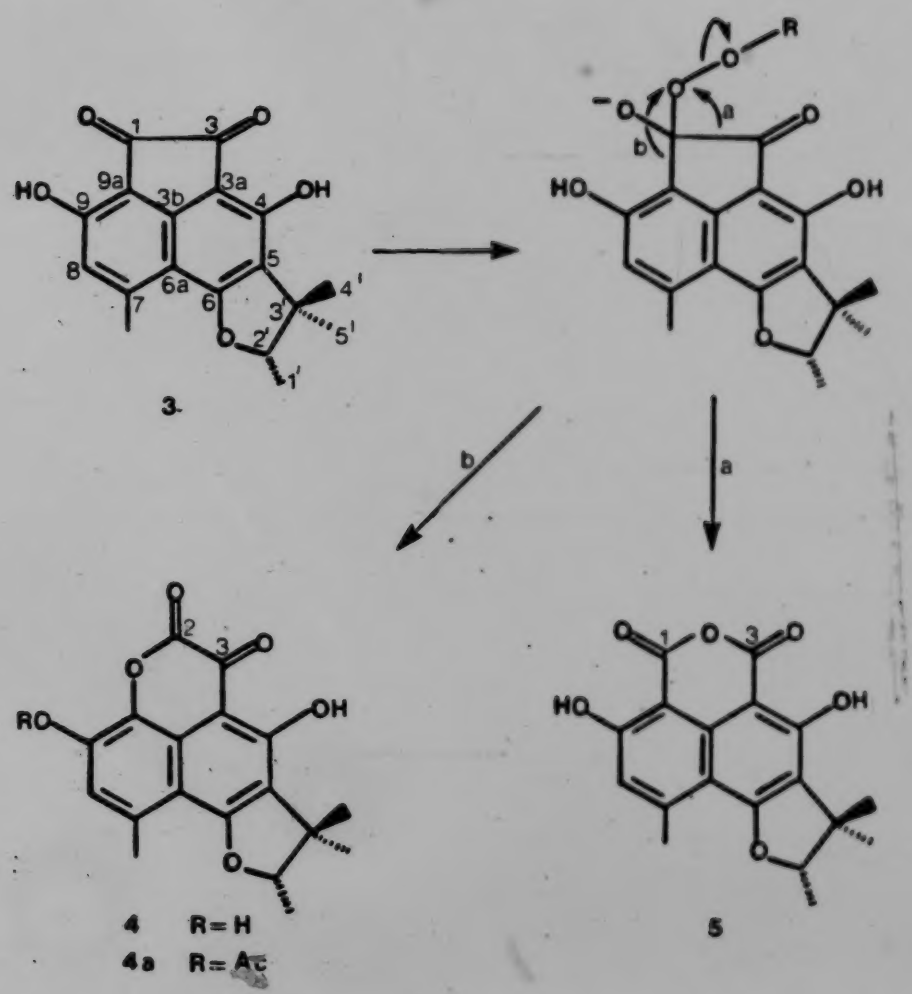


1

SCHEME IV-2



SCHEME IV-3

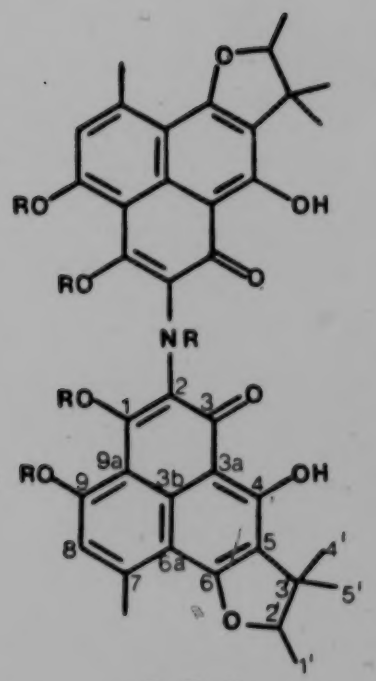


3

4 R=H
4a R=Ac

5

Scleroderris green (6).



6 R=H
6a R=Me

In order to follow the labelling pattern of ^{13}C enriched metabolites, it was first necessary to assign the chemical shifts of the ^{13}C nmr spectra. The fact that atrovenetinone (2) is produced in small amounts, is unstable in solution, and its ^{13}C nmr is difficult to analyze, prompted us to utilize compound 6a to represent metabolites with the intact phenalenone skeleton. Scleroderolide (4) was most conveniently isolated as the

monoacetate **4a**.

The ^{13}C nmr and ^{1}H nmr chemical shifts of **3**, **4a**, **5**, and **6a** are summarized in Tables IV-1 and IV-2. The ^{13}C nmr spectrum of **6a** was assigned previously.* Assignment of the ^{13}C nmr spectrum of sclerodin (**5**) was made as follows. The carbon shieldings in the proton decoupled spectrum may be sub-divided into three groups: 5 sp^2 carbons bound to oxygen (δ164-167); 7 other sp^2 carbons (δ150-93); 6 sp^3 carbons (δ14-93). The sp^3 carbons (C-1', C-2', C-3', C-4', C-5', and $ArCH_3$) and C-8 are readily assigned on the basis of chemical shift, multiplicity and by analogy to other metabolites (3). The remaining carbons of the phenalenone ring system are assigned by analysis of the 2, 3, and 4 bond ^{13}C-^{1}H coupling observed in the fully coupled spectrum (confirmed by selective proton decoupling experiments). Since the C-8 hydrogen should have large (ca. 6 Hz) (5) coupling to both C-6a and C-9a and small (ca. 2 Hz) (5) coupling to C-7 and C-9, while only C-6a and C-7 should be coupled to the $ArCH_3$, the assignment of C-6a, C-7, C-9, and C-9a can be made unambiguously. The phenolic hydrogens at C-9 and C-4 are strongly hydrogen bonded and show coupling to C-1, C-9a,

*For details, see Part 3 of this series.

Table IV-1. ^{13}C Chemical Shifts[a] of Some Metabolites and Derivatives of G. abietina.

Compound	3	4a	5	6a
C-1	189.8[x]		165.4	157.2
C-2		155.1		133.7
C-3	186.3[x]	170.7	164.9	177.2[x]
C-3a	106.2	107.6	93.5	107.7
C-3b	150.9	122.2	135.4	127.6
C-4	154.8	167.8	164.2	175.7[x]
C-5	119.6	121.1	119.1	119.3
C-6	164.3	169.0	166.1	166.5
C-6a	109.2	113.5	108.5	109.3
C-7	146.4	135.8	149.8	143.0
C-8	117.4	122.3	117.3	110.4
C-9	154.3	137.9	166.0	160.5
C-9a	107.2	135.0	97.3	110.7
$ArCH_3$	22.0	22.3	23.6	23.9
C-1'	14.5	14.6	14.5	14.7
C-2'	92.0	93.0	92.1	91.1
C-3'	43.3	43.4	43.5	43.4
C-4'	25.8	25.6	25.6	25.9
C-5'	21.0	20.5	20.7	20.5
$OO\underline{C}CH_3$		168.2		
$OOC\underline{C}H_3$		20.6		
OCH_3				56.4, 61.3
NCH_3				41.1

[x]Carbon assignments may be interchanged.

[a]In $CDCl_3$.

Table IV-2. ^1H nmr Data[a] for Some Metabolites and Derivatives of G. abietina.

Compound	3	4a	5	6a
H-4' (5')	1.29	1.35	1.32	1.32
H-1'	1.48 (d, 8.0)	1.52 (d, 6.5)	1.51 (d, 7.0)	1.46 (d, 6.5)
H-5' (4')	1.54	1.57	1.55	1.54
$ArCH_3$	2.75	2.79	2.84	2.87
H-2'	4.67 (q, 8.0)	4.80 (q, 6.5)	4.72 (q, 7.0)	4.64 (q, 6.5)
H-8	6.66	7.01	6.85	6.82
OH	7.48	13.77	11.43	18.50
	7.79		11.64	
Other		2.43 ($OCCH_3$)		3.68 (NCH_3)
				3.90 (OCH_3)
				4.03 (OCH_3)

[a]Shift (δ), (multiplicity, J (Hz)), in $CDCl_3$.

C-9 and C-3, C-3a, C-4, respectively.* The previous assignment of C-9a allows unambiguous assignment of the phenolic hydrogens (C-9 OH, δ11.43; C-4 OH, δ11.64). Of the carbons coupled to the C-4 OH group, C-3a is assigned on the basis of chemical shift while C-4 is distinguished from C-3 on the basis of the magnitude of the coupling constants ($^2J_{CH}$ = 5 Hz, $^4J_{CH}$ = 2 Hz). Similarly, for the carbons coupled to the C-9 OH group the previous assignment of C-9a and C-9 leads to the recognition of C-1 (confirmed by the magnitude of the coupling constants $^2J_{CH}$ = 5 Hz, $^4J_{CH}$ = 2 Hz). The remaining sp^2 carbon bound to oxygen is thus assigned as C-6 (confirmed by $^3J_{CH}$ to the C-2' H). Of the two remaining carbons, C-3b is assigned due to its lack of long range coupling, whereas C-5 appears as a multiplet in the coupled spectrum.

The assignment of the ^{13}C nmr spectrum of sclerodione (**3**) was made in a similar fashion. However, in this case the phenolic hydrogens appear as very broad signals in the ^1H nmr spectrum (δ7.48 and 7.79, $W_{1/2}$ = 16 Hz), precluding selective decoupling. Thus C-1 and C-3 are distinguished from C-4 and C-9 on the basis of chemical shift. While the assignment of C-9 (as with 5) allows the assignment of

*Additional coupling to C-8 and C-5 is apparent.

C-4, C-1 and C-3 cannot be assigned unambiguously.

The assignment of the ^{13}C nmr spectrum of scleroderqlide acetate (**4a**) was made in a similar manner as for sclerodin (**5**). The acetate carbonyl (δ168.2) is assigned by its two bond ^{13}C-^{1}H coupling to the acetyl methyl hydrogens (δ2.43). The C-2 carbonyl is assigned on the basis of its chemical shift (6) and its lack of long range coupling.

Gremmeniella abietina was grown in liquid still culture in the presence of sodium[1-^{13}C]acetate and, separately, in the presence of sodium [2-^{13}C]acetate. Compounds **3**, **4a**, **5**, and **6a** were isolated by chromatography.

Examination of the proton decoupled ^{13}C nmr spectrum of compound **6a** isolated from the culture containing [^{13}C-1]-labelled acetate, showed enrichment (about 5% incorporation) at carbons 1, 3, 3b, 4, 6, 7, 9, 1' and 3' relative to the natural abundance spectrum. Alternatively, the use of [^{13}C-2]-labelled acetate led to enrichment at the remaining carbons (C-2, C-3a, C-5, C-6a, C-8, C-9a, $ArCH_3$, C-2', C-4', and C-5'). These results are consistent with those obtained with P. herquei (3) and confirm the polyketide-mevalonate origin of these metabolites.

Examination of the proton decoupled ^{13}C nmr spectrum of sclerodione (**3**), isolated from the culture containing

[1-^{13}C] acetate, revealed the same labelling pattern as that of **6a**; again carbons* 1, 3, 3b, 4, 6, 7, 9, 1', and 3' were enriched (about 3% incorporation) relative to the natural abundance spectrum. When sclerodione (**3**) was isolated from the culture containing [2-^{13}C] acetate, carbons 3a, 5, 6a, 8, 9a, $ArCH_3$, 2', 4', and 5' were enriched. These results are fully consistent with the hypothesis for formation of sclerodione (**3**) by loss of C-2 from an intact phenalenone skeleton.

According to Scheme IV-3, oxidation of sclerodione (**3**), enriched at C-1 and C-3, would lead to sclerodin (**5**) also enriched at C-1 and C-3 (path a) and to scleroderolide (**4**) enriched at C-2 and C-3 (path b). The ^{13}C nmr spectrum of sclerodin (**5**), isolated from the culture containing [1-^{13}C] acetate, showed the same labelling pattern as that for **3** and **6a** (i.e., C-1 and C-3 are enriched). The ^{13}C nmr spectrum for scleroderolide acetate (**4a**) isolated from the same culture, however, failed to show the expected enrichment at C-2. These results were confirmed by incorporation of [2-^{13}C] acetate (C-2 of **4a** is derived from C-2 of acetate). Thus sclerodione (**3**) may be a biosynthetic precursor to

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*For biogenetic reasons (see below) the ketonic carbonyls are numbered 1 and 3.

sclerodin (**5**) but not to scleroderolide (**4**) (Scheme IV-4). The pathway leading to scleroderolide (**4**) must involve oxidation at an earlier stage, perhaps prior to cyclization of the heptaketide chain. Interestingly, *in vitro* oxidation of sclerodione (**3**) with m-chloroperbenzoic acid gives both scleroderolide (**4**) and sclerodin (**5**); however air oxidation gives only sclerodin (**5**).*

The oxidation of atrovenetin (**7**) to atrovenetinone (*ent*-**2**) (7) and 5 (8), as well as other unidentified products (9) has been reported. In our hands a dichloromethane-methanol solution of atrovenetin (**7**),** after standing for five weeks in the presence of air, gave rise to the enantiomers of compounds **2**, **3**, and **5** as the major products.

Examination of the hrms of crude methanol extracts of *G. abietina* revealed the presence of an ion corresponding to atrovenetin ($C_{19}H_{18}O_6$). Methylation (excess diazomethane) of the crude extract followed by repeated chromatography, provided the enantiomer of atrovenetin trimethyl ether (*ent*-**7a**), identical (tlc, ir ^1H nmr, ^{13}C

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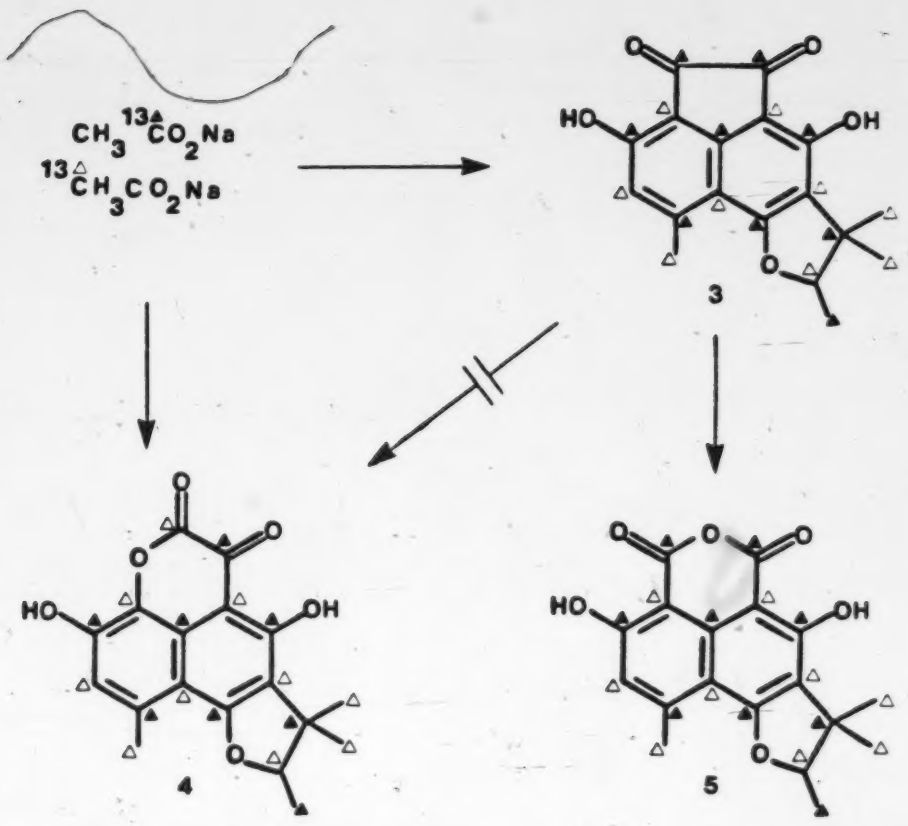
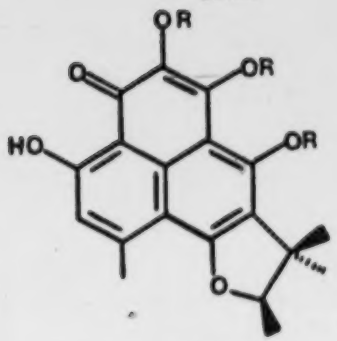
*For details, see Part 2 of this series.

**Sample provided by Professor L.C. Vining.

SCHEME IV-4





7 R=H
7a R=Me

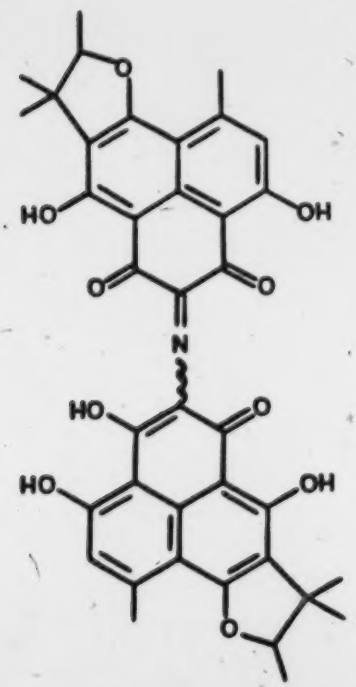
nmr, and hrms) with an authentic sample.*

The presence of the enantiomer of atrovenetin (7) in cultures of G. abietina raises the possibility that 2, 3, and 5 are produced by in vitro aerial oxidation. The possibility that the anhydride 5 is an isolation artifact has been mentioned previously (11), although in other cases (12) it seems to be of biological origin. Although the formation of some of compounds 2, 3, and 5 during the isolation process cannot be excluded, it seems unlikely that this is

*Prepared by methylation of atrovenetin (10). This reaction gives two isomers of atrovenetin trimethyl ether (see Experimental). Structures 7a and 9 were assigned by analysis of ^{13}C nmr spectra, as described for 6a.

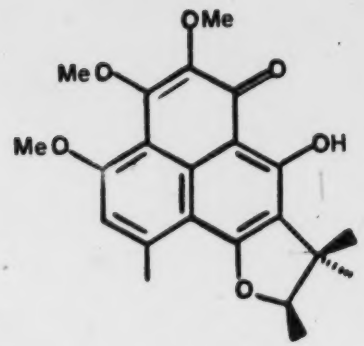
the only source of these substances; the air oxidation of 3 or 7 is a relatively slow process. It would appear that G. abietina catalyzes the oxidation of atrovenetin to other metabolites.

Another point that deserves consideration is the biogenesis of compound 6. According to Narasimhachari and Vining (7) the blue green pigments of P. herquei did not form when sodium sulphite was present in the culture medium. This blue pigment possesses structure 8 (1),



8

which may be formed by a ninhydrin-like reaction between atrovenetinone (**2**) and amino acids (1). Compound **6**, a reduced form of **8**, might be formed from atrovenetin (**7**) and atrovenetinone (**2**), *via* atrovenetinone transamination. At this stage we do not know whether both **6** and **8** are produced *in vivo* by *G. abietina*. It is possible that **8** is an artifact derived from **6**.



9

EXPERIMENTAL

All solvents except diethyl ether were distilled prior to use. ACS quality anhydrous diethyl ether was used without purification. Petroleum ether refers to Skelly Oil Company light petroleum, bp 62-72°C. Pyridine was distilled from CaH_2 and stored over molecular sieves, acetic anhydride was dried over P_2O_5 and distilled from sodium acetate. Sodium [1-^{13}C] acetate and sodium [2-^{13}C] acetate (90% enriched) were obtained from Merck, Sharp and Dohme Canada Ltd.

Analytical thin layer chromatography (tlc) was carried out on aluminum sheets (75 × 25 or 75 × 50 mm) pre-coated (0.2 mm) with silica gel $60F_{254}$ (E. Merck, Darmstadt). Preparative thin layer chromatography (ptlc) was carried out on glass plates (20 × 20 cm) pre-coated (0.25 mm) with the same adsorbent. Materials were detected by visualization under an ultraviolet (uv) lamp (254 or 350 nm), or by spraying with a solution of phosphomolybdic acid (5%) containing a trace of ceric sulfate, in aqueous sulfuric acid (5% v/v), followed by charring on a hot plate. Flash column chromatography (13) was performed with Merck Silica Gel (40-63 μm).

High resolution mass spectra (hrms) were recorded on

an A.E.I. MS-50 mass spectrometer coupled to a DS 50 computer. Data is reported as m/z (relative intensity). Infrared (ir) spectra were recorded on a Nicolet 7199 FT interferometer. ^{1}H nuclear magnetic resonance (^{1}H nmr) spectra and ^{13}C nuclear magnetic resonance (^{13}C nmr) spectra were measured on a Bruker WH-400 spectrometer. Solutions of 0.5-5% (w/v) in $CDCl_3$ (0.5 mL, 5 mm tube) were employed depending on availability of sample. For ^{1}H nmr, residual $CHCl_3$ in $CDCl_3$ was employed as the internal standard (assigned as 7.26 ppm downfield from tetramethylsilane (TMS)) and measurements are reported in ppm downfield from TMS (δ). For ^{13}C nmr, $CDCl_3$ was employed as the internal standard (assigned as 77.0 ppm downfield from TMS) and measurements are reported in ppm downfield from TMS (δ).

Incorporation of ^{13}C-labelled acetate

G. abietina C699 was grown in liquid still culture (10 L) in a medium of 10% V-8 juice and 1% glucose (14) in 2.8 L Fernbach flasks (1 liter of medium per flask), as reported previously.* After 15 days of growth, a sterile solution of sodium [1-^{13}C] acetate (0.082 g, 1 mmol) in

*For details, see Experimental, Part 2 of this series.

water (5 mL) was injected into each flask. After a further 7, and then 14 days, an additional 1.0 mmol of labelled acetate was injected into each flask. After a total 38 days of growth the mycelium was removed by filtration (through cheesecloth), washed with cold methanol and air dried in the dark (2 days). The dried mycelium was extracted in a Soxhlet extractor, twice with dichloromethane (12 and 24 h) and then twice with methanol (24 h each). The crude extracts were evaporated to dryness *in vacuo*, at room temperature. Compounds **3**, **4a**, and **5** were isolated from the first crude dichloromethane extract (*ca.* 6.0 g), whereas compound **6a** and **7a** were isolated from the second crude methanol extract (*ca.* 250 mg).

The dichloromethane extract (700 mg) was fractionated by flash column chromatography (dichloromethane-methanol-acetic acid, 48:1:1, 3 L) and the fractions (125 mL) were coevaporated with toluene. The fractions were further purified as indicated for the individual components.

The second crude methanol extract showed two major components on tlc; one component corresponding to Scleroderris green (**6**) (higher R_f) and the other to atrovenetin (**7**).

The experiments with sodium [2-^{13}C] acetate were carried out in the same way as described above for sodium

[1-^{13}C] acetate. The yields of the crude extracts were ca. 50% of the yields reported above.

Isolation of sclerodione (3)

Sclerodione (3) was eluted in fraction 5 (200 mg from 2.1 g of crude extract). Flash column chromatography (dichloromethane-methanol-acetic acid, 48:1:1) of this fraction gave crude sclerodione. Recrystallization from dichloromethane-petroleum ether gave sclerodione (20 mg), identical in all respects with an authentic sample.

Isolation of scleroderolide monoacetate (4a)

Scleroderolide (4) was more conveniently isolated as its monoacetate derivative 4a. Scleroderolide was eluted in fractions 6 and 7 in a very crude form. Flash column chromatography (dichloromethane-methanol-acetic acid, 48:1:1) of these combined fractions (300 g, from 2.1 g of crude extract) gave crude scleroderolide (25 mg). Acetylation (acetic anhydride/pyridine/4-N,N-dimethylaminopyridine), followed by ptlc (benzene-acetone-acetic acid, 90:10:0.1, double elution) gave scleroderolide monoacetate (5 mg), identical in all respects with an authentic sample.

Isolation of sclerodin (**5**)

Sclerodin (**5**) was eluted in fractions 2 and 3. Flash column chromatography (petrolum ether-ethyl acetate, 4:1) of these combined fractions (600 mg, from 1.4 g of crude extract) gave crude sclerodin. Recrystallization from dichloromethane-petroleum ether gave sclerodin (20 mg), identical in all respects with an authentic sample.

Isolation of pentamethyl Scleroderris green (**6a**)

Pentamethyl Scleroderris green (**6a**) was obtained on methylation (ethereal diazomethane) of the methanol extract. After 12 h the crude reaction mixture was evaporated to dryness and fractionated twice by ptlc (petroleum ether-ethyl acetate, 4:1, multiple elution) to give compound **6a** (ca. 2 mg from 100 mg of crude extract), identical in all respects with an authentic sample.

Isolation of atrovenetin trimethyl ether (ent-**7a**)

Atrovenetin trimethyl ether (ent-**7a**) was obtained on methylation (excess ethereal diazomethane) of the methanol extract. After 12 h fractionation of the crude reaction mixture by ptlc (petroleum ether-ethyl acetate, 4:1, multiple elution) gave a yellow residue (yellow fluorescence under uv light). Further purification by ptlc (benzene-acetone, 1:1) gave compound ent-**7a** ($[\alpha]_D$

-103°) identical with an authentic sample (prepared from atrovenetin as follows) by tlc, ir, ^{1}H nmr, ^{13}C nmr, and hrms comparison.

Methylation of atrovenetin (7)

Atrovenetin* (ca. 16 mg) was suspended in dichloromethane and excess ethereal diazomethane was added at room temperature. After 12 hours the reaction mixture was evaporated and fractionated by ptlc (benzene-acetone, 19:1, double elution), to afford two major products **7a** (ca. 4 mg, 22% yield) and **9** (ca. 8 mg, 44% yield).

Atrovenetin trimethyl ether (**7a**)

tlc: R_f 0.35 (benzene-acetone, 19:1), yellow spot (yellow fluorescence under uv light); ir ($CHCl_3$, cast): 3240 (br), 2920, 1610, 1585, 1460, 1320, 1285 cm^{-1}; ^{1}H nmr ($CDCl_3$): δ1.28 (3H, s, C-3' CH_3), 1.45 (3H, d, 7.0 Hz, C-2' CH_3), 1.54 (3H, s, C-3' CH_3), 2.80 (3H, s, $ArCH_3$), 3.85 (3H, s, OCH_3), 4.03 (3H, s, OCH_3), 4.09 (3H, s, OCH_3), 4.60 (1H, q, 7.0 Hz, C-2' H), 6.84 (1H, s, C-8 H), 17.67 (1H, s, OH); ^{13}C nmr ($CDCl_3$): δ14.4 (C-1'), 22.1, (C-4'), 26.2 (C-5'), 24.5 ($ArCH_3$), 44.3 (C-3'), 61.0, 62.3, 64.4 ($3\times OCH_3$), 90.5 (C-2'), 108.4 (C-6a), 109.3 (C-9a),

*Sample provided by Professor L. Vining.

109.9 (C-3a), 121.3 (C-8), 126.9 (C-5), 127.8 (C-3b), 140.3 (C-2), 149.9 (C-7), 157.6 (C-4), 160.5 (C-3), 163.3 (C-6), 174.7 (C-9), 177.3 (C-1);* hrms: m/z calcd. for $C_{22}H_{24}O_6$: 384.1572; found: 384.1560(100), 369(79), 341(67), 311(13).

Atrovenetin trimethyl ether (**9**)

tlc: R_f 0.32 (benzene-acetone, 19:1), yellow spot; ir ($CHCl_3$, cast): 3240 (br), 2920, 1610, 1585, 1455, 1355, 1300, 1260, 1220 cm^{-1}; ^1H nmr ($CDCl_3$): δ1.33 (3H, s, C-3' CH_3), 1.48 (3H, d, 7.0 Hz, C-2' CH_3), 1.57 (3H, s, C-3' CH_3), 2.90 (3H, s, $ArCH_3$), 4.05 (6H, s, OCH_3), 4.11 (3H, s, OCH_3), 4.68 (1H, q, 7.0 Hz, C-2' H), 6.88 (1H, s, C-8 H), 18.10 (1H, s, OH); ^{13}C nmr ($CDCl_3$): δ14.6 (C-1'), 20.5 (C-4'), 25.9 (C-5'), 24.1 ($ArCH_3$), 43.3 (C-3'), 56.5, 60.8, 61.4 ($3\times OCH_3$), 91.4 (C-2'), 108.2 (C-3a), 109.4 (C-6a), 109.6 (C-9a), 110.7 (C-8), 119.4 (C-5), 127.3 (C-3b), 142.4 (C-2), 144.7 (C-7), 157.3 (C-1), 161.2 (C-9), 166.9 (C-6), 173.9 (C-3), 176.0 (C-4); hrms: m/z calcd. for $C_{22}H_{24}O_6$: 384.1572; found: 384.1577(50), 369(100), 351(9), 341(8).

*Assignments for C-3 and C-4 as well as C-1 and C-9 may be interchanged.

Aerial oxidation of atrovenetin (**7**)

Atrovenetin (**7**, 20 mg) was suspended in chloroform-methanol (1:1) and exposed to sunlight in a loosely stoppered flask. After 5 weeks separation by ptlc (dichloromethane-methanol, 49:1, double elution) of the reaction mixture gave three major fractions: R_f 0.90 (3 mg, 16%), R_f 0.55 (5 mg, 25%), and R_f 0.12 (4 mg, 22%). The R_f 0.90 compound was identical with sclerodin (**5**) by tlc, ^1H nmr, and hrms comparison. The R_f 0.55 compound was identical with atrovenetinone (**2**) by tlc and hrms comparison; its ethanolate (hot ethanol) was also identical with atrovenetinone ethanolate (**2a**) by ir comparison. The R_f 0.12 compound was identical with sclerodione (**3**) by tlc, ^1H nmr, ^{13}C nmr, and hrms comparison.

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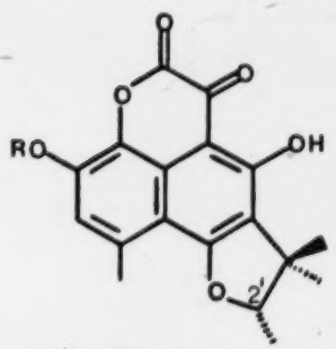
V. GENERAL DISCUSSION

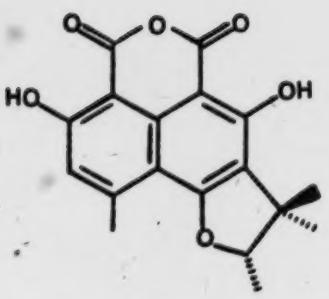
The Scleroderris canker fungus, Gremmeniella abietina grown in liquid culture produces an array of colorful metabolites (1). One of these metabolites is a bright yellow compound which we have named scleroderolide. Scleroderolide (1) was isolated from the mycelium extract of G. abietina strain C699 (so-called "European strain", from New Brunswick). The structure of this metabolite was determined by X-ray crystallographic analysis of its monoacetate 1a. The sample of scleroderolide monoacetate



1 R=H
1a R=Ac

(1a) used in the X-ray crystallographic study was racemic, though the parent compound (1) was optically active ($[\alpha]_D$ -22.3°). Surprisingly, scleroderolide isolated more recently from a fresh culture of G. abietina has a much higher optical rotation ($[\alpha]_D$ -116°). The apparent

inconsistency of these results has no simple explanation. Experiments performed with scleroderolide (**1**), the monoacetate **1a**, and two structurally related metabolites, sclerodin (**2**) and sclerodione (**3**), indicated



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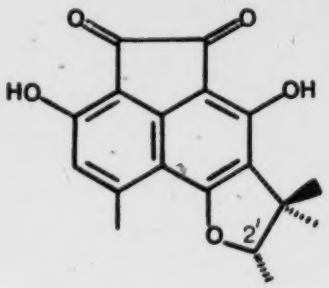

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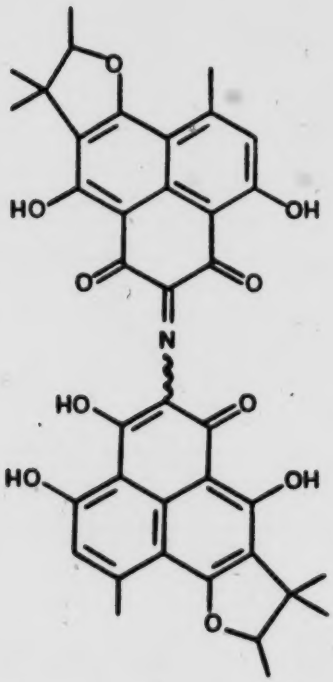
that racemization does not occur during isolation of **1**, or during the preparation of the acetate **1a**. Furthermore, scleroderolide isolated from other fresh cultures of *G. abietina* (C699) consistently has the same optical rotation ($[\alpha]_D$ -116°). It is possible that the initially isolated scleroderolide (**1**, $[\alpha]_D$ -22.3°) was produced by a mutant of strain C699. We also cannot exclude the possibility that the strain used in the experiment yielding the partially racemic material was misidentified.

The configuration at C-2' of scleroderolide (**1**, levorotatory) was determined by correlation of **1** with sclerodione (**3**). Peracid oxidation of sclerodione (levorotatory, S-configuration (1) at C-2') provides levorotatory scleroderolide (**1**), establishing the S-configuration at C-2'.

The most recently isolated metabolite from G. abietina strain C699 is a rather unstable, yellowish green compound, which on standing is transformed into Scleroderris blue (**4**). The structure of this compound (**5**)



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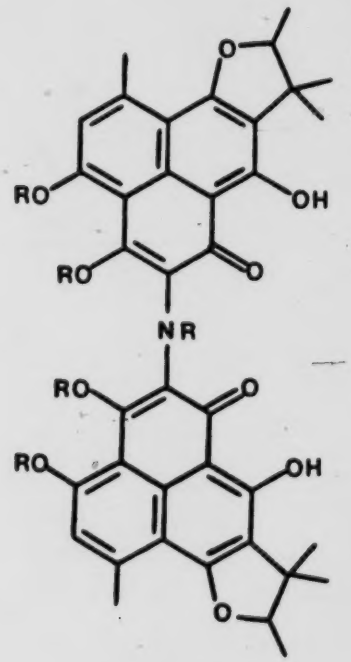
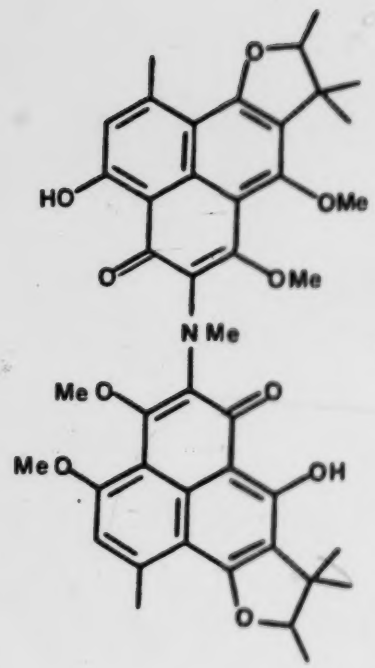


5 R=H
5a R=Me

was assigned by analysis of the spectroscopic data of the three isomeric pentamethyl derivatives 5a, 6, and 7



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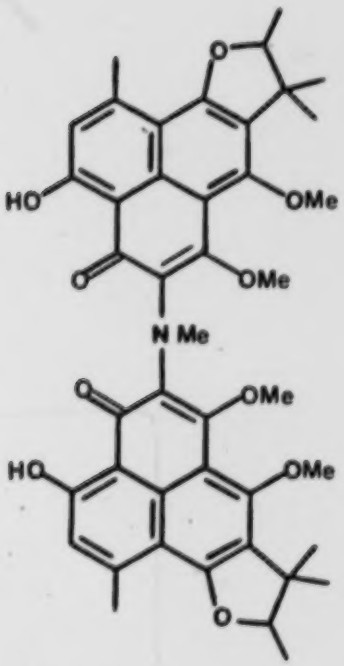

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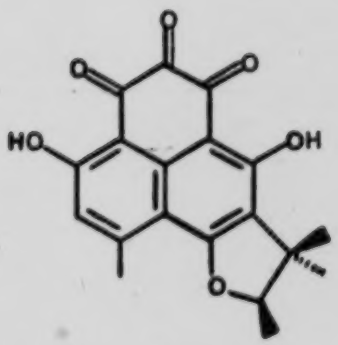
obtained on treatment with diazomethane. Compound 5, named Scleroderris green, is the reduced form of Scleroderris blue (4). This structural relationship was confirmed by treatment of 4 with sodium bisulphite to obtain 5. Earlier studies (2) on the metabolites of Penicillium herquei showed that the blue-green pigmentation in the mycelium did not appear when sodium sulphite was added to the culture medium. It was also suggested (2) that this blue-green pigment might be formed by a ninhydrin-like reaction between atrovenetinone (8)



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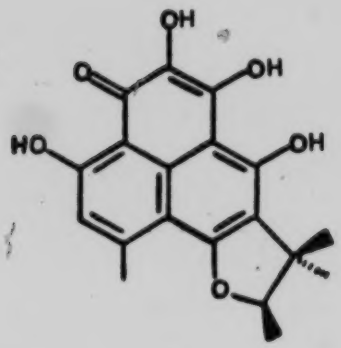
and amino-acids, although the structure of the pigment was not assigned. Later studies (1) showed that Scleroderris blue (4) could be obtained by treatment of atrovenetinone

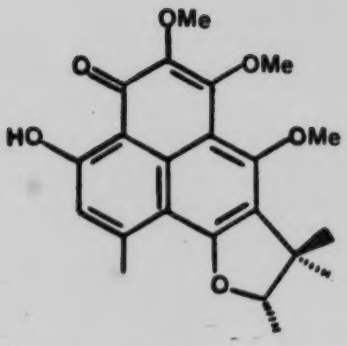
with glycine; it was then suggested that the blue-green pigment of *P. herquei* had structure 4. In view of these reports we suggest that Scleroderris green (5) may be formed in a similar way, by reaction of atrovenetin (9)



9

and atrovenetinone (8) with amino acids, and that it may contribute to the pigmentation of the diseased wood.

Considering the close structural relationship between some of the metabolites produced by *G. abietina* and the phenalenones of the atrovenetin (9) type, we suspected that atrovenetin (or its enantiomer) might also be produced by *G. abietina*. The trimethyl ether of *ent*-atrovenetin (10) was isolated from a crude extract of strain C699 after treatment with diazomethane. Thus the



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enantiomer of atrovenetin (**9**) is apparently produced by G. abietina, although in relatively small amounts. Since in vitro aerial oxidation of atrovenetin (**9**) gives compounds **8**, ent-**2**, and ent-**3**, it is possible that they are artifacts of isolation. However, since this oxidation process is relatively slow and these compounds are obtained in much greater amounts than atrovenetin, we believe that they are true metabolites of G. abietina.

The crude extracts of different strains of G. abietina (from North America, Europe, and Japan) were compared by high-pressure liquid chromatography. Two of the strains isolated in Canada (C659 and C699) had similar chromatographic profiles, while the third (C656) was quite

different. Following these observations, the metabolites of G. abietina C656 (Alberta) were examined. The major metabolites produced by this strain were fatty acids and triglycerides; some minor sterols were also isolated. However, no phenalenones could be detected in the crude extract. Considering the differences in the metabolites produced by the strain from Alberta and the strains of G. abietina previously studied, a question is raised regarding the classification of this strain. Strain differences have been indicated by pathogenicity tests (3) and cultural characteristics (4). The fungus isolated in Alberta is apparently a milder pathogen (5). These reports and our results may indicate a correlation between the pathogenicity of G. abietina isolates and the phenalenone metabolites and raise several interesting questions. Since strain C656 does not produce phenalenones, does it still produce a yellowish green discoloration of the inner bark and wood of the trees? Does the only other strain (C654) of G. abietina isolated in Alberta resemble strain C656, or does it produce phenalenones?

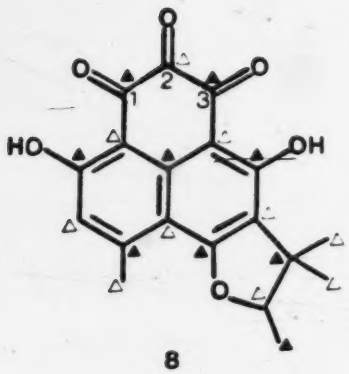
Studies on the metabolites of Penicillium herquei have established the polyketide origin of the C_{14} phenalenone nucleus and the derivation of the C_5 side chain from mevalonate (6). Subsequently the folding of

the precursor heptaketide chain was determined by ^{13}C studies (7). We have studied the biosynthesis of the metabolites produced by G. abietina and confirmed the polyketide origin of the C_{14} (and derived C_{13}) phenalenone nucleus. The results obtained through incorporation of [^{13}C-1]- and [^{13}C-2]-labelled acetate are summarized in Scheme V-1. The labelling pattern obtained in sclerodione (**3**, i.e. C-1 and C-3) can be rationalized in terms of its derivation from triketone **8** by oxidative loss of C-2 (8). Further oxidation of sclerodione (**3**) can lead to the anhydride **2**. Unexpectedly (8), the results show that sclerodione is not the biogenetic precursor of scleroderolide (**1**). The pathway leading to scleroderolide must involve oxidation at an earlier stage, perhaps prior to cyclization of the heptaketide chain.

SCHEME V-1

$CH_3{}^{13\Delta}CO_2Na$

$^{13\Delta}CH_3CO_2Na$



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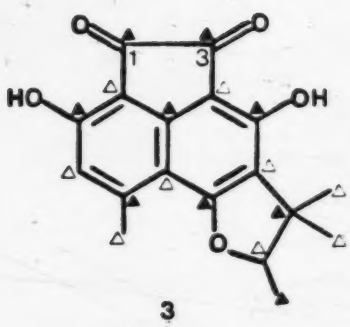

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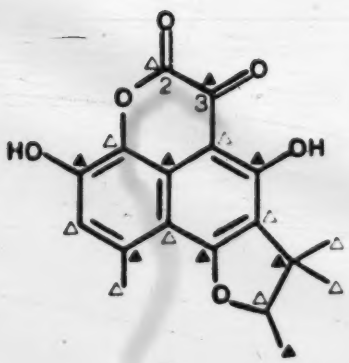

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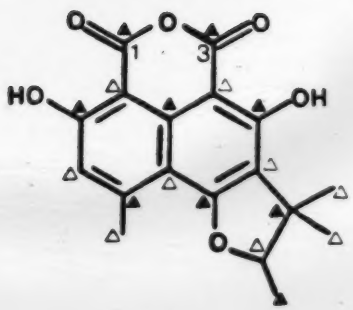

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APPENDIX 1*

X-RAY CRYSTALLOGRAPHIC DATA OF SCLERODEROLIDE MONOACETATE.

*The X-ray structure determination was carried out by J. Clardy and E. Arnold, Department of Chemistry, Cornell University, Ithaca, N.Y.

Red-orange single crystals of racemic scleroderolide monoacetate suitable for X-ray diffraction analysis were obtained from CH_2Cl_2-Skelly B. Preliminary X-ray photographs showed triclinic symmetry. Precise lattice constants of a = 11.027(2), b = 9.716(1), c = 9.300(1)Å, α = 77.44(1), β = 91.34(1) and χ = 117.97(1)°, were determined by a least squares analysis of the diffractometrically measured angular positions of 15 reflections. The results of the X-ray analysis show the space group for the crystals to be $P\bar{1}$, in which one molecule of scleroderolide monoacetate (MW = 370.44, V = 855.4(2)$Å^3$, ρ_{calc} = 1.44 g/cm^{-3}) forms the asymmetric unit. All unique diffraction data with $2\theta < 114°$ were surveyed using Nickel-filtered CuKα radiation (λ = 1.54178Å) and a variable-speed 1° ω-scan on a Syntex $P2_1$ diffractometer. After correction for Lorentz, polarization, and background effects, 1957 of a total of 2300 unique data (85.1%) were considered observed ($|F_o| > 3\sigma_F$) and used for the ensuing structure solution and refinement.

The structure of scleroderolide monoacetate was solved by standard multisolution direct methods.* All hydrogens were located by a difference synthesis following partial refinement. Block-diagonal least squares refinement, with anisotropic oxygens and carbons and isotropic hydrogens, converged to a residual of .047 for the observed data.

Figure A1-1 shows a computer-generated perspective drawing of the final X-ray model of one enantiomer of scleroderolide monoacetate. The five-membered ring of scleroderolide monoacetate is in the C_2 conformation with

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*All crystallographic calculations were done on a PRIME 850 computer, operated by the Cornell Chemistry Computing Facility. Principal programs employed were REDUCE and UNIQUE, data reduction programs: Leonowicz, M.E., Cornell University, 1978; MULTAN78, A System of Computer Programs for the Automatic Solution of Crystal Structures from X-ray Diffraction Data, direct methods programs and Fast Fourier Transform routine (locally modified to perform all Fourier calculations including Patterson syntheses): Main, P.; Hull, S.E.; Lessinger, L.; Germain, G.; Declercq, U.-P.; Woolfson, M.M., University of York, England, 1978; NQEST, CYBER 173 version, negative quartets figure of merit calculation: Weeks, C.M., Medical Foundation of Buffalo, Inc., August 1976; BLS78A, anisotropic block-diagonal least squares refinement: Hirotsu, K.; Arnold, E., Cornell University, 1980; ORTEP, crystallographic illustration program: Johnson, C.K., Oak Ridge, ORNL-3794; For a summary description of MULTAN, see: Germain, G.; Main, P.; Woolfson, M.M., Acta Crystallogr. Sect. B **1970**, B26, 274-285. Woolfson, M.M., Acta Crystallogr. Sect. A **1977**, A33, 219-225. For a summary of NQEST, refer to: De Titta, G.T.; Edmonds, J.W.; Langs, D.A.; Hauptman, H., Acta Crystallogr. Sect. A **1975**, A31, 472-479.

the two-fold axis bisecting the C5-C6 bond. There is an intramolecular hydrogen bond between O4(H) and O3 with an O4(H)-O3 distance of 1.57Å and OHO angle of 156°. The best least squares plane through the 13 atoms of the three six-membered rings shows a maximum deviation of 0.095Å from the plane for atom C9. Although the substituents O3, O4, O6, and C3' are also within 0.09Å of this plane, O2, C7Me, and O9, are respectively 0.20, 0.16, and 0.18Å away from this plane.

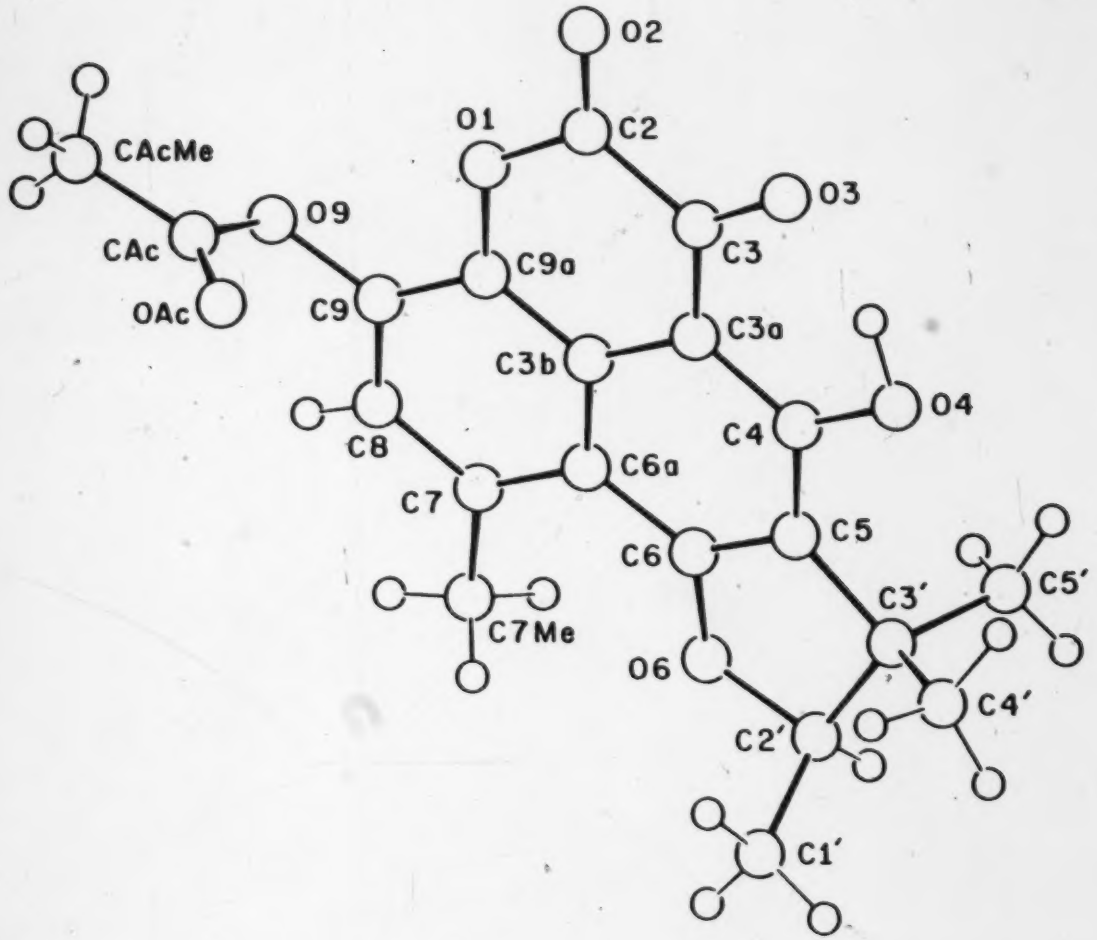

Figure A1-1. A computer generated perspective drawing of the X-ray model of scleroderolide monoacetate. The sample used for X-ray analysis was racemic.

Table A1-1

Fractional coordinates and thermal parameters for Scleroderolide monoacetate

Standard deviations of the least significant figures are given in parentheses. The isotropic equivalent thermal parameter is given for anisotropic atoms (denoted by an asterisk).

Atom	x	y	z	B
O1	0.1088(2)	0.2940(2)	0.0080(2)	4.1(1)*
C2	0.1087(3)	0.2752(3)	0.1577(3)	4.0(2)*
C3	-0.0186(3)	0.2505(3)	0.2427(3)	3.7(2)*
C3a	-0.1243(3)	0.2637(3)	0.1614(3)	3.4(2)*
C3b	-0.1110(3)	0.2976(3)	0.0031(3)	3.4(2)*
C4	-0.2438(3)	0.2430(3)	0.2366(3)	3.6(2)*
C5	-0.3475(3)	0.2566(3)	0.1558(3)	3.6(2)*
C6	-0.3293(3)	0.2956(3)	0.0034(3)	3.7(2)*
C6a	-0.2123(3)	0.3180(3)	-0.0822(3)	3.5(2)*
C7	-0.1967(3)	0.3488(3)	-0.2388(3)	3.8(2)*
C8	-0.0841(3)	0.3501(3)	-0.3024(3)	4.0(2)*
C9	0.0137(3)	0.3270(3)	-0.2189(3)	3.7(2)*
C9a	0.0034(3)	0.3063(3)	-0.0687(3)	3.7(2)*
C1'	-0.6588(4)	0.1001(5)	0.0317(4)	6.4(4)*
C2'	-0.5480(3)	0.2535(4)	0.0571(3)	4.6(2)*
C3'	-0.4814(3)	0.2481(3)	0.2046(3)	4.0(2)*
O6	-0.4346(2)	0.3100(3)	-0.0592(2)	4.6(1)*
O2	0.2079(2)	0.2800(3)	0.2141(2)	5.2(2)*
O3	-0.0213(2)	0.2227(2)	0.3796(2)	4.6(2)*
O4	-0.2574(2)	0.2109(2)	0.3840(2)	4.2(1)*
O9	0.1242(2)	0.3322(2)	-0.2941(2)	4.3(1)*
OAc	0.0347(3)	0.0675(3)	-0.2133(3)	6.2(2)*
CAc	0.1194(3)	0.1877(4)	-0.2890(3)	4.7(2)*
CAc'Me	0.2330(4)	0.2076(4)	-0.3880(4)	5.7(2)*
C7Me	-0.2966(3)	0.3769(4)	-0.3372(3)	4.8(2)*
C4'	-0.5691(3)	0.1024(4)	0.3294(4)	5.5(2)*
C5'	-0.4496(3)	0.4014(4)	0.2565(4)	5.3(2)*
H8	-0.070(3)	0.371(3)	-0.403(3)	5.3(7)
H1'	-0.746(3)	0.056(4)	0.097(4)	7.6(9)
H1'	-0.691(4)	0.103(4)	-0.062(4)	8.2(9)
H1'	-0.616(5)	0.024(6)	0.047(5)	11.9(13)
H2'	-0.583(3)	0.346(3)	0.033(3)	4.9(6)
H7Me	-0.387(3)	0.284(4)	-0.318(4)	6.9(8)
H7Me	-0.270(3)	0.395(4)	-0.440(4)	7.2(8)
H7Me	-0.304(4)	0.474(4)	-0.321(4)	7.3(8)
H4'	-0.398(3)	0.404(4)	0.178(4)	7.1(8)
H4'	-0.527(3)	0.409(4)	0.283(3)	6.4(8)
H4'	-0.395(3)	0.411(4)	0.347(4)	7.3(9)
H5'	-0.572(3)	0.011(3)	0.305(3)	4.6(6)
H5'	-0.521(3)	0.112(4)	0.430(3)	5.8(7)
H5'	-0.663(3)	0.087(4)	0.343(3)	6.2(7)

Table A1-1 (Continued)

Atom	x	y	z	B
H09	-0.166(4)	0.209(4)	0.411(4)	8.7(10)
HAc11e	0.262(3)	0.127(3)	-0.360(3)	5.4(7)
HAc11e	0.315(4)	0.284(5)	-0.377(4)	9.0(10)
HAc11e	0.215(4)	0.230(4)	-0.482(4)	7.3(9)

Table A1-2

Bond distances for Scleroderolide monoacetate

The standard deviation of the least significant figure of each distance is given in parentheses.

O1	- C2	1.365(3)	C6	- O6	1.338(4)
O1	- C9a	1.388(4)	C6a	- C7	1.419(4)
C2	- C3	1.519(5)	C7	- C8	1.381(5)
C2	- O2	1.187(4)	C7	- C7Me	1.500(5)
C3	- C3a	1.415(4)	C8	- C9	1.389(4)
C3	- O3	1.241(3)	C9	- C9a	1.367(4)
C3a	- C3b	1.433(3)	C9	- O9	1.394(4)
C3a	- C4	1.414(4)	C1'	- C2'	1.483(5)
C3b	- C6a	1.418(4)	C2'	- C3'	1.544(4)
C3b	- C9a	1.400(4)	C2'	- O6	1.482(4)
C4	- C5	1.396(4)	C3'	- C4'	1.530(4)
C4	- O4	1.334(3)	C3'	- C5'	1.546(5)
C5	- C6	1.379(4)	O9	- CAc	1.370(5)
C5	- C3'	1.515(5)	OAc	- CAc	1.176(3)
C6	- C6a	1.434(4)	CAc	- CAcMe	1.484(6)

Table A1-3

Bond angles for Scleroderolide monoacetate

The standard deviation of the least significant figure of each angle is given in parentheses.

C2	- O1	- C9a	122.2(2)
O1	- C2	- C3	117.8(3)
O1	- C2	- O2	118.5(3)
C3	- C2	- O2	123.7(3)
C2	- C3	- C3a	118.3(2)
C2	- C3	- O3	117.2(3)
C3a	- C3	- O3	124.5(3)
C3	- C3a	- C3b	120.3(3)
C3	- C3a	- C4	120.0(2)
C3b	- C3a	- C4	119.7(3)
C3a	- C3b	- C6a	121.9(3)
C3a	- C3b	- C9a	118.8(3)
C6a	- C3b	- C9a	119.3(2)
C3a	- C4	- C5	119.7(2)
C3a	- C4	- O4	120.3(3)
C5	- C4	- O4	120.1(3)
C4	- C5	- C6	119.2(3)
C4	- C5	- C3'	131.3(2)
C6	- C5	- C3'	109.3(3)
C5	- C6	- C6a	125.0(3)
C5	- C6	- O6	112.7(3)
C6a	- C6	- O6	122.3(2)
C3b	- C6a	- C6	114.4(2)
C3b	- C6a	- C7	120.3(3)
C6	- C6a	- C7	125.2(3)
C6a	- C7	- C8	117.5(3)
C6a	- C7	- C7Me	123.6(3)
C8	- C7	- C7Me	118.9(2)
C7	- C8	- C9	122.3(3)
C8	- C9	- C9a	120.4(3)
C8	- C9	- O9	117.7(2)
C9a	- C9	- O9	121.8(3)
O1	- C9a	- C3b	122.1(2)
O1	- C9a	- C9	117.9(3)
C3b	- C9a	- C9	120.0(3)
C1'	- C2'	- C3'	117.5(3)
C1'	- C2'	- O6	105.9(3)
C3'	- C2'	- O6	105.2(2)
C5	- C3'	- C2'	101.2(2)
C5	- C3'	- C4'	114.0(3)
C5	- C3'	- C5'	109.3(2)
C2'	- C3'	- C4'	114.9(2)
C2'	- C3'	- C5'	108.5(3)
C4'	- C3'	- C5'	108.5(3)
C6	- O6	- C2'	108.9(2)

Table A1-3 (Continued)

C9	- O9	- CAc	116.4(2)
O9	- CAc	- OAc	122.0(4)
O9	- CAc	- CAcMe	111.0(2)
OAc	- CAc	- CAcMe	127.0(4)

APPENDIX 2

CHROMATOGRAPHIC ANALYSIS OF CRUDE EXTRACTS OF *GREMMENIELLA ABIETINA*.

Seven strains of the fungus Gremmeniella abietina were grown in still culture* in a medium of 10% (v/v) clarified V-8 juice containing 1% (w/v) added glucose:

C656 - North American strain, from Alberta
C659 - North American strain, from Ontario
C699 - European strain, from New Brunswick
C704 - Asian strain, from Japan
C706 - European strain, from Sweden
C707 - New York strain, from New York
C708 - New York strain, from Vermont

After four weeks of growth, strains C659 and C699 have a very similar appearance, dark green mycelia with bright yellow spots floating on a clear greenish broth, whereas strain C708 has a more brownish mycelia and broth. However strains C656, C704, C706, and C707 were quite different, having a whitish-grey mycelium mat floating on a clear yellowish broth.

These strains (except C708) were grown on agar plates containing a medium of 10% (v/v) clarified V-8 juice with 1% (w/v) added glucose. After four weeks the content of

*For details, see Chapter II.

each plate was extracted with methanol (20 mL, 3 days) and this was filtered through a plug of cotton wool. High-pressure liquid chromatography (hplc) analysis was carried out on these crude extracts.

The chromatographic analyses were performed under the following conditions:*

Column:	μBondapak C_{18} Radial-PAK Cartridge (8 mm × 10 cm)
Mobile phase:	CH_3OH (containing 1% (v/v) of CH_3CO_2H)-H_2O (4:1)
Flow rate:	2.5 mL/min
Run volume:	50 mL
Detection:	254 nm
Injection volume:	15-20 μL

The crude extracts of strains C656 and C707 had similar chromatographic profiles, showing no distinct peaks (Figure A2-1). The metabolite spectra of strains C659 and C699 are similar, although quantitative differences exist; both strains show peaks with the same retention time as sclerodione (about 4.6 min),

*The hplc equipment is described in Chapter III.

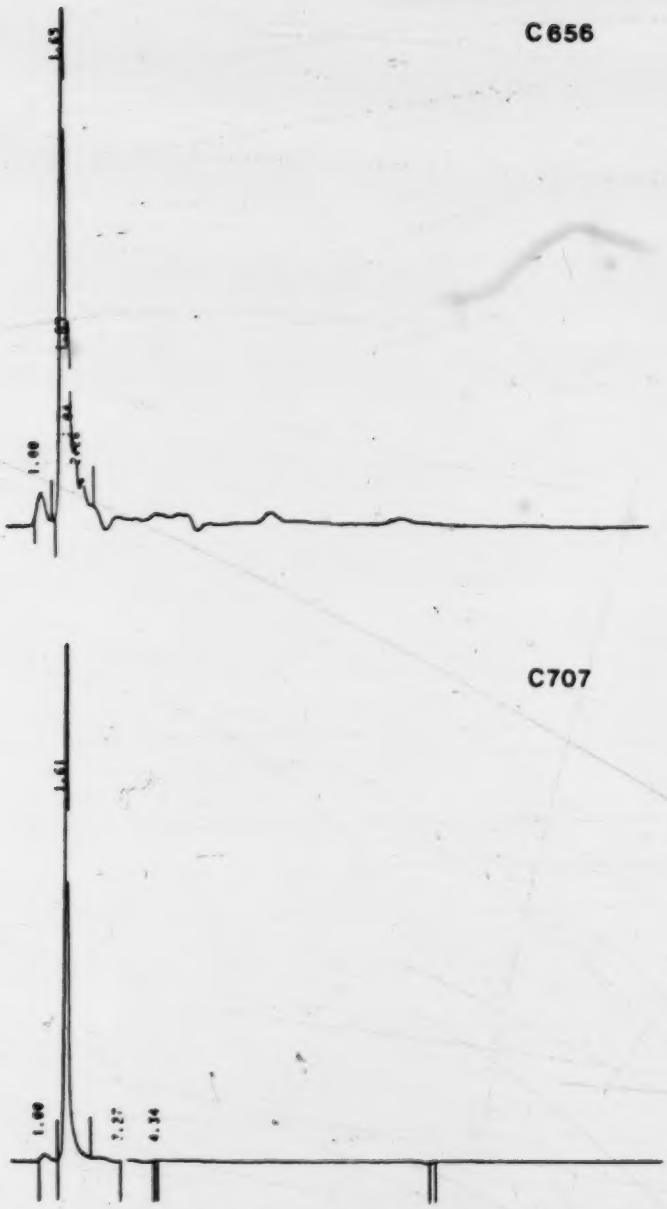

Figure A2-1. Chromatograms of crude extracts of strains C656 and C707.

scleroderolide (about 5.4 min), and sclerodin (about 11.6 min). Other peaks present (Figure A2-2) could not be assigned. Strains C704 and C706 have different chromatographic profiles from the other four strains (Figure A2-3); both show a peak with the same retention time as sclerodin (about 11.6 min).

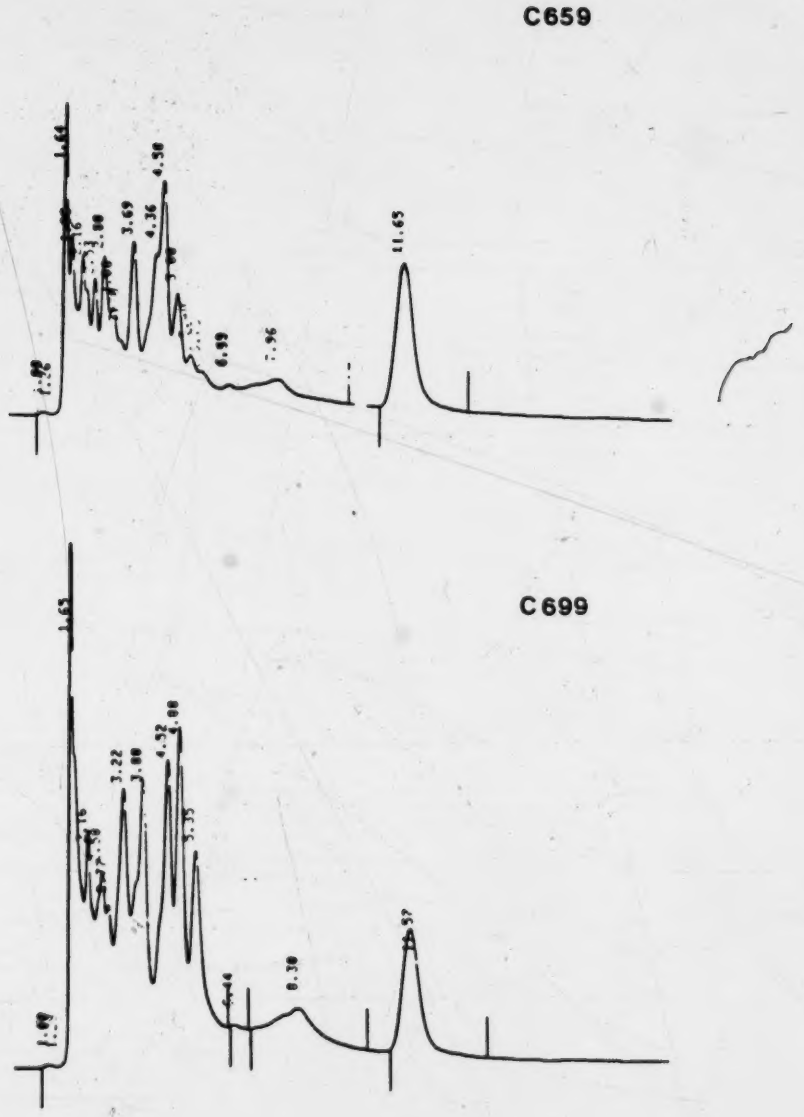

Figure A2-2. Chromatograms of crude extracts of strains C659 and C699.

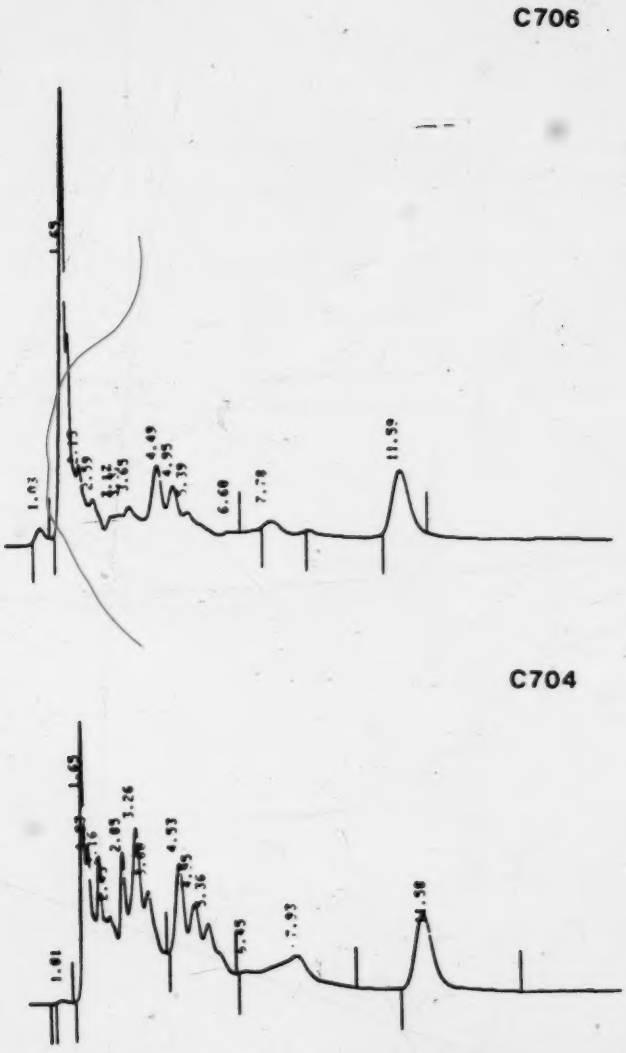

Figure A2-3. Chromatograms of crude extracts of strains C704 and C706.

APPENDIX 3

BIOLOGICAL ACTIVITY OF CRUDE EXTRACTS OF *GREMMENIELLA ABIETINA*.

The biological activity of crude extracts of the Scleroderris canker fungus Gremmeniella abietina was tested against several bacteria. Two different bioassays were used: an agar diffusion assay (Kirby-Bauer) and a turbidimetric assay (complemented with a surface plate count).

The crude mycelium extracts (dichloromethane, ether or methanol) of strain C699 are a dark green powder, very soluble in dimethylsulphoxide (DMSO) and sparingly soluble in water. The tests were carried out with the ether soluble portion of the crude extracts.

In the Kirby-Bauer bioassay, filter paper discs soaked with the crude extract solution (0.8 mg/mL in CH_2Cl_2:MeOH, 2:1) were placed on the surface of an agar medium (Mueller Hinton) containing a standardized suspension of bacteria. After 24 hours of incubation (36 ±1°C) the surface of the agar plates was examined. The surface of the agar acquired a turbid appearance (a positive result is recorded when transparent haloes (inhibition zone) remain around the filter paper discs). A solvent blank and standard concentrations of an antibiotic (Penicillin G 10 units and Cephalothin 30 µg, Sensitivity Discs, Difco Laboratories) were used to compare the relative antibacterial activity of the crude extracts. The inhibition zone diameters obtained with the

crude extracts were very small (<0.2 mm) as compared with Penicillin G (>22 mm) or Cephalothin (> 18 mm). In addition, this zone showed no coloration, indicating that the colored metabolites present in the crude extracts of G. abietina have a relatively slow diffusion rate into the agar medium. When Tween 20 was added to the crude extract solutions, the diameter of the inhibition zone did not change significantly (<0.4 mm). For this reason the turbidimetric method described next was examined.

Turbidimetric Assay

The bacterial inoculum was prepared by inoculating 10 mL of sterile media (Mueller Hinton) with three standard discs (Bacto Disk Set A, Difco Laboratories). After 24 hours of incubation (35 ± 1°C) the inoculum was shaken and used immediately. Sterile trypticase soy medium (5.0 mL in Spectronic 20 tubes) was inoculated with about three drops* of each bacterial inoculum. The crude extract solution (16.2 mg/mL in DMSO, 50 μL) was added to the inoculated medium and the content of each tube was shaken. The transmitance (T) of each tube was recorded

*The turbidity of the bacterial culture was compared with a standardized suspension of $BaSO_4$ (prepared from 0.5 mL of 1.175% (w/v) $BaCl_2 \cdot H_2O$ and 99.5 mL 1% (w/v) H_2SO_4).

(600 nm) in a Spectronic 20 spectrophotometer (calibrated to 100% T with a solution prepared in the same way but omitting bacteria). A control test was carried out substituting the crude extract solution for DMSO (50 μL). After 24 h of incubation (with shaking) the transmittance (%) was recorded. The results reported in the following table represent the difference (Δ) in T(%) between the tubes containing the bacterial media with the crude extract solution and the tubes containing the same bacterial media (DMSO does not inhibit the growth of the microorganism).

In order to demonstrate that changes in T(%) were due to differences in bacterial concentrations rather than effects of the colored metabolites, bacterial concentrations of control and test solutions for the same microorganisms were confirmed by surface plate counts.*

An antibacterial substance can inhibit the growth of a bacterial population, either by killing all the cells (bactericidal) or by inhibiting the capacity of each individual cell to duplicate (bacteriostatic). A single

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*H.W. Seeley, Jr. and P.J. VanDemark. Microbes in Action, 2nd Edition, W.H. Freeman and Company, San Francisco, 1972. pp 51-57.

Table A3-1. Bioactivity of crude extracts of *Gremmeniella abietina*.

Microorganism	Δ^a %T at 24 h
Enterobacter cloacae	25
Escherichia coli	15
Klebsiella pneumoniae	15
Proteus vulgaris	<5
Pseudomonos aeruginosa	25
Salmonella typhimurium	15
Serratia marcescens	15
Staphylococcus aureus	15
Staphylococcus epidermis	15
Streptococcus pyogenes	15

[a] Δ %T is the difference in transmitance (%T) between the tubes containing the bacterial media with the crude extract solution and the tubes containing the bacterial media, after 24 hours of incubation.

antibiotic may be both bacteriostatic and bactericidal, depending on the concentration used. The crude extracts of the fungus G. abietina apparently exhibit some bacteriostatic activity. Whether this bioactivity is the result of the inhibition of the microorganism growth by a single or by several compounds has not been determined.